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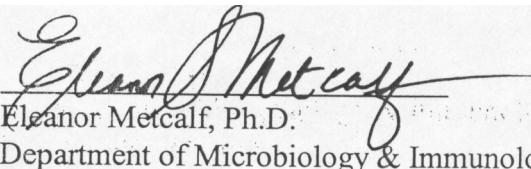
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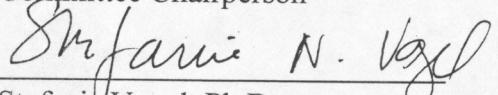
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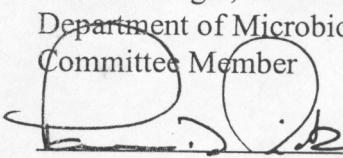
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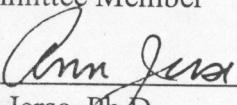
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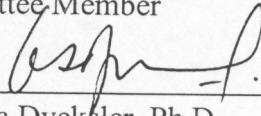
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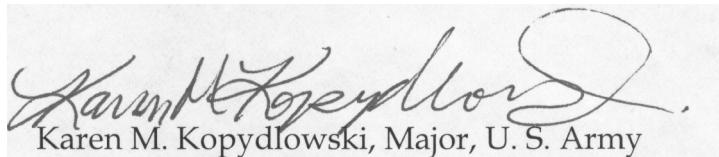
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## ABSTRACT

Title of Dissertation: Regulation of Chemokine Expression by  
Lipopolysaccharide *In Vitro* and *In Vivo*

Karen M. Kopydlowski, Doctor of Philosophy, 2002

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Microbiology and Immunology, Uniformed Services University of the Health  
Sciences

The host response to Gram negative LPS is characterized by an influx of inflammatory cells into host tissues which is mediated predominantly by the localized production of chemokines. The influx and activation of inflammatory leukocytes, in combination with their overproduction of proinflammatory mediators, are believed to result in the tissue damage that precedes the multiple organ failure and death associated with sepsis. The overall goal of this work was to identify cellular and molecular mechanisms that contribute to the differential regulation of chemokine expression in response to LPS, in an effort to enhance our understanding of the unique roles of individual chemokines in a disease process as complex as sepsis. Our *in vitro* examination of LPS-inducible chemokine genes revealed variability in the magnitude and kinetics of

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REGULATION OF CHEMOKINE EXPRESSION BY LIPOPOLYSACCHARIDE  
*IN VITRO AND IN VIVO*

By

Karen M. Kopydlowski

Dissertation submitted to the Faculty of the  
Department of Microbiology and Immunology Graduate Program of the  
Uniformed Services University of the Health Sciences  
In partial fulfillment for the degree of  
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Before completing my degree, I was assigned to the Walter Reed Army Institute of Research in the Division of Experimental Therapeutics, where I was tasked with establishing a Malaria Microarray Program for Drug Discovery. My ability to straddle two worlds and manage to succeed in both was largely attributable to truly supportive friends and colleagues. First and foremost, I would like to extend my deepest appreciation to Dr. Sheila A. Peel for establishing an open and collaborative environment for the co-development of the Microarray Program, for intensive training in the fields of molecular parasitology and DNA microarrays, and for professional and personal mentorship and guidance. Together, we have succeeded in overcoming the most insurmountable odds and have made great strides in developing a first-class program. To my Department Chief, Dr. Thomas Hudson, thank you for

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## **DEDICATION**

In loving memory of my father, Donald J. Kopydlowski.

## TABLE OF CONTENTS

	<b>INTRODUCTION</b>	<b>PAGE</b>
	Sepsis and the Role of Lipopolysaccharide	1
	LPS Recognition and Signaling	5
	The Inflammatory Response to LPS	14
	Properties of the Chemokine Superfamily	20
	Chemokine Subfamilies	25
	Chemokine Receptors	34
	Chemokine Receptor Signaling	37
	Chemokines in Leukocyte Trafficking	41
	Redundancy versus Specificity	46
	Thesis Rationale	51
	<b>MATERIALS AND METHODS</b>	
	Reagents	53
	Mice	54
	Mouse Models	57
	Macrophage Isolation and Culture Conditions	57
	Isolation of Total Cellular RNA	58
	Reverse Transcription-Polymerase Chain Reaction	58
	Detection and Quantitation of PCR Products	60

Blood Collection and Preparation for Analysis	62
Chemokine and Cytokine ELISAs	62
Detection of Nitric Oxide (NO)	64
Flow Cytometric Analysis of Bone Marrow, Thymocytes, and Splenocytes	64
Myeloperoxidase Assay	65
Isolation of Hepatic Leukocytes	66
Flow Cytometric Analysis of Hepatic Leukocytes	66
NK Cell-Enriched Cultures for IFN- $\gamma$ Production	67
Statistics	68
<b>III. RESULTS</b>	
<b>A. In Vitro Analysis of LPS-Induced Chemokine Expression in Murine Peritoneal Macrophages</b>	69
Kinetic analysis of CXC and CC chemokine mRNA expression induced by LPS and MPL	70
Dose response analysis of CXC and CC chemokine mRNA expression induced by LPS and MPL	73
Induction of CXC and CC chemokine mRNA by macrophage activating agents other than LPS does not require a normal <i>Lps</i> gene product	76
Suppression of LPS-induced chemokine mRNA expression and protein secretion in LPS-tolerant macrophages	80
IL-10-mediated suppression of LPS-induced chemokine mRNA expression	85
Selective inhibition of LPS-induced chemokine mRNA expression and protein production by tyrphostin AG556	85

Selective inhibition of LPS-induced chemokine mRNA expression by IFN- $\gamma$	93
Induction of IP-10 and MCP-5 mRNA by LPS or LPS and IFN- $\gamma$ is Stat1-dependent	93
IFN- $\gamma$ -mediated inhibition of LPS-induced KC mRNA is dependent on IRF-2	97
<b>B. In Vivo Analysis of LPS-Induced Chemokine Expression</b>	103
Kinetic Analysis of CXC and CC chemokine mRNA expression in tissue after administration of LPS	103
Kinetic analysis of chemokine levels in the serum after administration of LPS	107
Effect of macrophage depletion on chemokine mRNA expression in the liver	107
<b>C. Analysis of the Role of KC in a Murine Model of LPS-Induced Lethality</b>	113
Targeted disruption of the mouse N51/KC gene	114
Reduction of LPS-induced mortality in KC <sup>-/-</sup> mice	117
Biochemical analysis of indices of liver injury	121
Evaluation of circulating leukocytes and neutrophil infiltration into tissue	121
Kinetic analysis of the predominant neutrophil chemoattractants, KC and MIP-2, after administration of LPS	127
Kinetic analysis of proinflammatory and anti-inflammatory cytokines after administration of LPS	131
Examination of upstream mediators of IFN- $\gamma$ production: IL-12, IL-18 and IL-15	139

Evaluation of inducible nitric oxide synthase, an IFN- $\gamma$ -dependent mediator of LPS-induced pathogenesis	143
Analysis of cell sources of IFN- $\gamma$	143
IFN- $\gamma$ production by NK cells	146
<b>IV. DISCUSSION</b>	
<i>In Vitro</i> Chemokine Regulation in Murine Peritoneal Macrophages	152
<i>In Vivo</i> Chemokine Regulation in Response to LPS	163
The Role of KC in a Murine Model of LPS-Induced Lethality	166
Summary of Work and Future Perspectives of Chemokines as Targets for Therapeutic Intervention in Gram Negative Sepsis	183
<b>V. BIBLIOGRAPHY</b>	186

## LIST OF TABLES

	<b>PAGE</b>
Table I. PCR oligonucleotide primer and probe sequences, annealing temperatures, and cycle numbers	61
Table II. Production of JE/MCP-1, KC, MIP-2, and TNF- $\alpha$ in culture supernatants of LPS-tolerized macrophages	84
Table III. Effect of IL-10 on the production of JE/MCP-1 and MIP-2 in culture supernatants of LPS-stimulated macrophages	88
Table IV. Production of JE/MCP-1 and MIP-2 in culture supernatants of LPS-stimulated macrophages pretreated with tyrphostin AG556	92
Table V. Effect of IFN- $\gamma$ on the production of JE/MCP-1, MIP-1 $\alpha$ , and MIP-2 in culture supernatants of LPS-stimulated macrophages	96
Table VI. Effect of IFN- $\gamma$ on the production of KC in culture supernatants of LPS-stimulated macrophages of IRF-2 $^{+/+}$ and IRF-2 $^{-/-}$ mice	102
Table VII. Distribution of phenotypic markers on bone marrow, lymph node, and spleen cells from KC $^{+/+}$ and KC $^{-/-}$ mice	118
Table VIII. Serum AST and ALT levels in KC $^{+/+}$ and KC $^{-/-}$ mice following injection with a lethal dose of LPS	122
Table IX. Effects of a lethal dose of LPS on circulating leukocyte populations in KC $^{+/+}$ and KC $^{-/-}$ mice	124
Table X. Circulating IL-12 p70 and IL-18 in KC $^{+/+}$ and KC $^{-/-}$ mice following injection with a lethal dose of LPS	142

## LIST OF FIGURES

	PAGE
Figure 1. Diagram of Enterobacterial LPS (A) and Lipid A (B)	4
Figure 2. LPS Signaling	8
Figure 3. Physiological Consequences of Agonist LPS-Macrophage Interactions	18
Figure 4. The Chemokine Superfamily	23
Figure 5. The Role of Chemokines in Leukocyte Trafficking	43
Figure 6. Kinetics of LPS- and MPL-induced expression of CXC (A) and CC (B) chemokine mRNA	72
Figure 7. Dose response analysis of LPS- and MPL-induced expression of CXC (A) and CC (B) chemokine mRNA	75
Figure 8. Differential induction of CXC (A) and CC (B) chemokines in response to macrophage-activating agents in macrophages from C3H/OuJ ( <i>Lps<sup>n</sup></i> ) and C3H/HeJ ( <i>Lps<sup>d</sup></i> ) mice	78
Figure 9. Suppression of LPS-induced CXC (A) and CC (B) chemokine mRNA expression in LPS-tolerant macrophages	83
Figure 10. Suppression of LPS-induced chemokine mRNA expression by IL-10	87
Figure 11. Selective inhibition of LPS-induced chemokine mRNA expression by tyrphostin AG556	91
Figure 12. Selective inhibition of IFN- $\gamma$ on LPS-induced CC (A) and CXC (B) chemokine mRNA expression	95
Figure 13. The effect of Stat1 on the induction of LPS- and LPS plus IFN- $\gamma$ -induced mRNA expression	99
Figure 14. The effect of IRF-2 on the IFN- $\gamma$ -mediated inhibition of LPS-induced mRNA expression	101

Figure 15.	Kinetics of CXC (A) and CC (B) chemokine mRNA expression in the lung and liver of C57BL/6J mice after administration of LPS	106
Figure 16.	Production of MIP-2, MIP-1 $\alpha$ , and JE/MCP-1 in the serum of C57BL/6J mice after administration of LPS	109
Figure 17.	Kinetics of CXC (A) and CC (B) chemokine mRNA in the livers of control (open bars) and Kupffer cell-depleted (solid bars) C57BL/6J mice after administration of LPS (25 $\mu$ g)	112
Figure 18.	Targeted disruption of the mouse N51/KC gene	116
Figure 19.	Reduction of LPS-induced mortality in KC $^{-/-}$ mice	120
Figure 20.	Neutrophil sequestration in lung and liver of KC $^{+/+}$ and KC $^{-/-}$ mice injected i.p. with a lethal dose of LPS (35 mg/kg)	126
Figure 21.	Kinetics of KC and MIP-2 mRNA expression in the liver (A) and lung (B) and protein in serum (C) of KC $^{+/+}$ and KC $^{-/-}$ mice injected i.p. with a lethal dose of LPS (35 mg/kg)	129
Figure 22.	CXCR2 mRNA expression in the liver of LPS-injected KC $^{+/+}$ and KC $^{-/-}$ mice	133
Figure 23.	Kinetics of cytokine mRNA expression in the liver (A) and lung (B) of LPS-injected (35 mg/kg) KC $^{+/+}$ and KC $^{-/-}$ mice	136
Figure 24.	Production of IFN- $\gamma$ and TNF- $\alpha$ in the serum of KC $^{+/+}$ and KC $^{-/-}$ mice after administration of LPS	138
Figure 25.	Kinetics of IL-15 mRNA expression in the liver and lungs of LPS-injected (35 mg/kg) KC $^{+/+}$ and KC $^{-/-}$ mice	141
Figure 26.	Kinetics of iNOS mRNA expression in the lung (A) and serum nitrate/nitrite (B) of KC $^{+/+}$ and KC $^{-/-}$ mice injected i.p. with a lethal dose of LPS (35 mg/kg)	145
Figure 27.	Distribution of NK, NK/T, and T cells in the livers of KC $^{+/+}$ and KC $^{-/-}$ mice 6 h after administration of LPS (35 mg/kg)	148
Figure 28.	Production of IFN- $\gamma$ in supernatants of NK cell-enriched cultures derived from livers of KC $^{+/+}$ and KC $^{-/-}$ mice	150

## ABBREVIATIONS

-/-	homozygous disruption of a gene on both chromosomes
ALT	alanine aminotransferase
AST	aspartate aminotransferase
bp	base pairs
BPI	bactericidal permeability-increasing protein
BSA	bovine serum albumin
but-LPS	butanol-extracted LPS
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
Cl <sub>2</sub> MBP	dichloromethylene bisphosphonate
dNTPs	deoxynucleotide triphosphates
EDTA	disodium ethylene diamine tetraacetate • 2H <sub>2</sub> O
ELR	glutamate-leucine-arginine
ENA-78	epithelial cell-derived neutrophil attractant-78 amino acid
ERK	extracellular signal-regulated kinases
ES	embryonic stem
FBS/FCS	fetal bovine serum/fetal calf serum
GAPDH	glyceraldehyde-3-phosphate
GPCR	G protein coupled receptor
GRO- $\alpha/\beta/\gamma$	growth related oncogene
h	hour(s)

HBSS	Hank's buffered saline solution
HEPES	N-2-hydroxy-ethyl piperazine-N'-2ethanesulfonic acid
HPRT	hypoxanthine-guanine phosphoribosyl transferase
I-CAM	intercellular adhesion molecule
ICSBP	interferon consensus sequence binding protein
IFN	interferon
IFN- $\alpha/\beta/\gamma$	interferon-alpha/beta/gamma
IgG	immunoglobulin G
IL	interleukin
iNOS	inducible nitric oxide synthase
IP-10	interferon- $\gamma$ -inducible protein 10
IRAK	IL-1 receptor associated kinases
IRF	interferon regulatory factor
JE	murine CC chemokine family member
JNK	c-Jun N-terminal kinase
KC	murine CXC chemokine family member
kDA	kilodalton
KDO	2-keto-3-deoxyoctulosonic acid
LBP	lipopolysaccharide binding protein
LFA	lymphocyte function associated antigen
LIX	LPS-induced CXC chemokine
LPS	lipopolysaccharide

<i>lps</i>	lipopolysaccharide response gene
<i>lps<sup>d</sup></i>	lipopolysaccharide response gene, defective allele
<i>lps<sup>n</sup></i>	lipopolysaccharide response gene, normal allele
mM	millimolar
mAb	monoclonal antibody
Mal	MyD88-adaptor-like protein
MAP	mitogen activated protein
µg	microgram
µl	microliter
µM	micromolar
MCP	monocyte chemoattractant protein
MEK	MAPK/ERK kinase
MeXAA	5,6-dimethylxanthenone-4-acetic acid
MIG	monokine inducible by interferon- $\gamma$
min	minutes
MIP	macrophage inflammatory protein
M-MLV	Moloney Murine Leukemia Virus
MPL	monophosphoryl lipid A
MPO	myeloperoxidase
mRNA	messenger ribonucleic acid
MW	molecular weight
MyD88	myeloid differentiation factor 88

NF-κB	nuclear factor kappa-B
ng	nanogram
NIK	nuclear factor kappa-B inhibitory kinase
NK	natural killer
nm	nanometer
nM	nanomolar
NO	nitric oxide
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PF4	platelet factor 4
PI3K	phosphatidylinositol 3 kinase $\gamma$
PKA/B/C	protein kinase A/B/C
PLD	phospholipase D
PRR	pattern recognition receptor
RANTES	regulated upon activation normal T expressed and secreted
RNA	ribonucleic acid
RT	reverse transcription
SAPK	stress-activated protein kinase
SDS	sodium dodecyl sulfate
SEM	standard error of the mean

SSC	sodium chloride-sodium citrate buffer
STAg	soluble extract of <i>Toxoplasma gondii</i> tachyzoites surface tachyzoites antigen
Stat1	signal transducer and activator of transcription
TAB	TAK1 binding protein
TAE	tris-acetate-disodium ethylene diamine tetraacetate • 2H <sub>2</sub> O
TAK	transforming growth factor-β-activated kinase
Th1	T-helper cell type 1
Th2	T-helper cell type 2
TIR	Toll/IL-1 receptor
TIRAP	TIR domain containing adaptor protein
TLR	Toll-like receptor
TNF-α	tumor necrosis factor alpha
TRAF	TNF receptor-associated factor
UTR	untranslated region

## INTRODUCTION

### Sepsis and the Role of Lipopolysaccharide

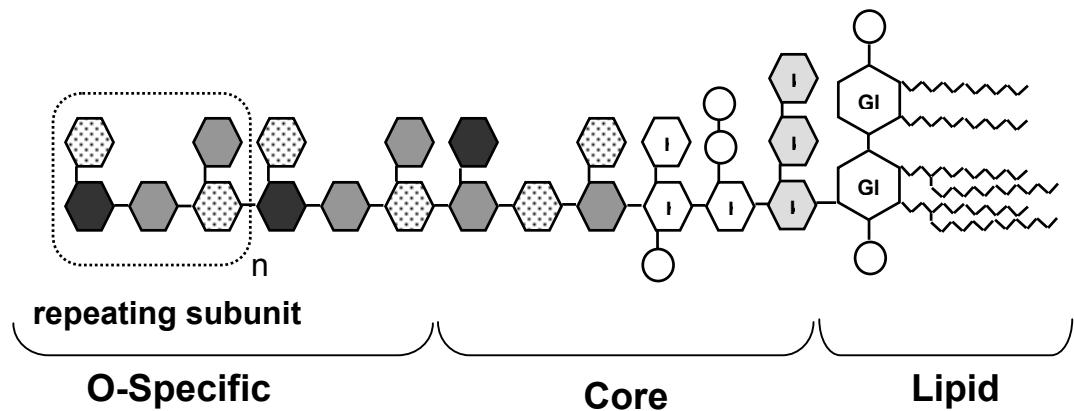
Despite recent advances in our understanding of the pathophysiologic mechanisms of sepsis and intensive efforts to develop an effective therapeutic strategy, sepsis remains a significant challenge for modern medicine. In the United States alone, over 750,000 cases of severe sepsis occur each year, with mortality rates ranging from 28% to 50% (1-3). Sepsis already ranks as the leading cause of death in intensive care units and its incidence is expected to increase as a result of the aging population, aggressive cancer therapies, invasive surgical devices, and widespread antibiotic resistance (4). Sepsis is defined as an infection-induced systemic inflammatory syndrome that is characterized by fever, hypotension, hypoglycemia, tachycardia, tachypnea, disseminated intravascular coagulation, and in severe cases, multiple organ failure and shock (5). The inflammatory cascade initiated during sepsis involves complex interactions among numerous mediators and physiologic systems. A greater understanding of these mediators and their complex interactions is necessary to fuel the development of novel strategies for the treatment of sepsis, as therapies directed at individual components of sepsis have proven largely unsuccessful (2, 3).

Among the most common causes of sepsis is bacterial infection, particularly by Gram negative aerobic bacteria (6). Implicated in triggering the inflammatory cascade associated with sepsis is lipopolysaccharide (LPS), an

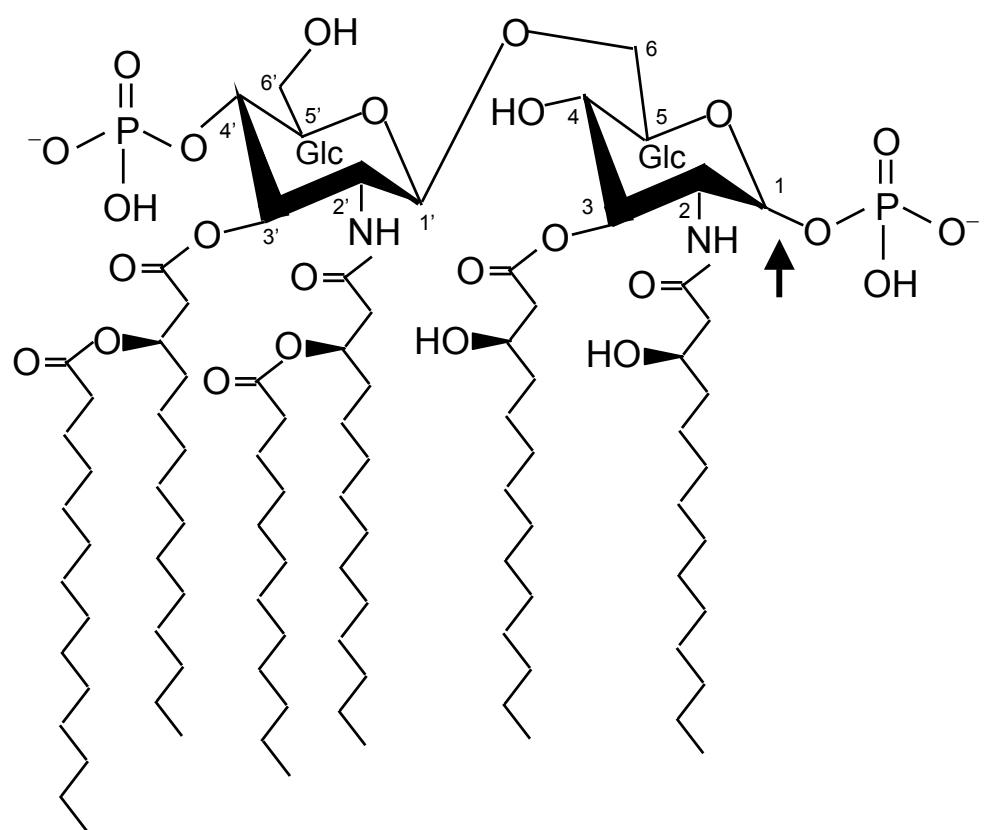
integral outer membrane component of Gram negative bacteria. Originally designated as "endotoxin" to distinguish it from secreted bacterial "exotoxins," LPS is a heat-stable, amphipathic molecule that is comprised of three structurally and functionally distinct regions: the O-specific chain, the core polysaccharide, and the lipid A domain (Figure 1A). The hydrophilic O-specific chain is a polymer of repeating oligosaccharide units that confers the smooth (S) colony morphology characteristic of Gram negative bacteria. Due to its structural variability among different Gram negative bacteria, the O-specific chain allows for the serological typing of individual strains according to O-antigenic determinants (7). Bacterial mutants that are defective in O-specific chain biosynthesis (*i.e.*, mutations or deletions in the *wb* or *rfl* gene locus) have truncated LPS structures, and consequently, are designated as "R"-mutants due to their rough phenotype (8). While not essential for viability, the O-specific chain is necessary for protection from phagocytosis and complement-mediated lysis *in vivo* (9, 10). Linking the O-specific chain to lipid A is a core polysaccharide region that is relatively conserved among bacterial families on the basis of its monosaccharide composition. Among the common elements in the enterobacterial outer core region are hexoses like D-glucose, D-galactose, and 2-amino-2-deoxy-D-glucose, while the inner core region contains heptose residues and 2-keto-3-deoxyoctulosonic acid (KDO), an essential component that is common to almost all bacterial LPS (11).

**Figure 1.** Diagram of Enterobacterial LPS (A) and Lipid A (B). (A) This schematic depicts the three structurally and functionally distinct regions of enterobacterial LPS. The O antigen is a polysaccharide comprised of repeating subunits ( $n = 1-50$ ) which confers the serological specificity, as well as the smooth or "S" phenotype, to Gram negative bacteria. The core region is an oligosaccharide that is highly conserved in its monosaccharide composition among bacterial species. The outer core is comprised primarily of hexoses, while the inner core contains heptoses (hep) and 2-keto-3-deoxyoctulosonic acid (KDO). Lipid A, the endotoxic moiety of LPS, is a glucosamine (GlcN) disaccharide that is diphosphorylated (P) and hexa-acylated. (B) Lipid A is a 1, 4'-bisphosphorylated  $\beta$  (1 $\rightarrow$ 6)-linked D-glucosamine disaccharide, which is acylated at positions 2, 2', 3, and 3' with variable length fatty acid chains. Two additional fatty acid chains are linked to the primary chains at positions 2' and 3' to produce a hexa-acylated structure predominantly. The arrow indicates the position of the phosphoryl group that is removed in monophosphoryl lipid A, the non-toxic derivative of Lipid A. Figures (A) and (B) are adapted from Alexander and Rietschel (11).

A.



B.



The hydrophobic lipid A domain is covalently-linked to a KDO residue of the inner core polysaccharide region via an acid-sensitive ketosidic bond. The highly conserved structure of enterobacterial lipid A consists of a 1, 4'-bisphosphorylated  $\beta$  (1 $\rightarrow$ 6)-linked D-glucosamine disaccharide, which is acylated at positions 2, 3, 2', and 3' with variable length fatty acid chains (Figure 1B) (11). Two additional fatty acid chains are linked to the primary chains at positions 2' and 3' to produce a hexa-acylated structure. Lipid A was proven to be the "endotoxic" principle of LPS when Galanos demonstrated that a polysaccharide-free, solubilized preparation of lipid A exhibited toxic and pyrogenic properties equivalent to the most potent LPS preparations (12). Further validation was achieved when chemically synthesized lipid A was demonstrated to exhibit comparable "endotoxic" activity as natural lipid A in numerous *in vitro* and *in vivo* assays (13, 14). Extensive investigations with synthetic and natural lipid A partial structures concluded that a complete lipid A structure (*i.e.*, hexaacylated and bisphosphorylated) was required for maximal biological activity (15).

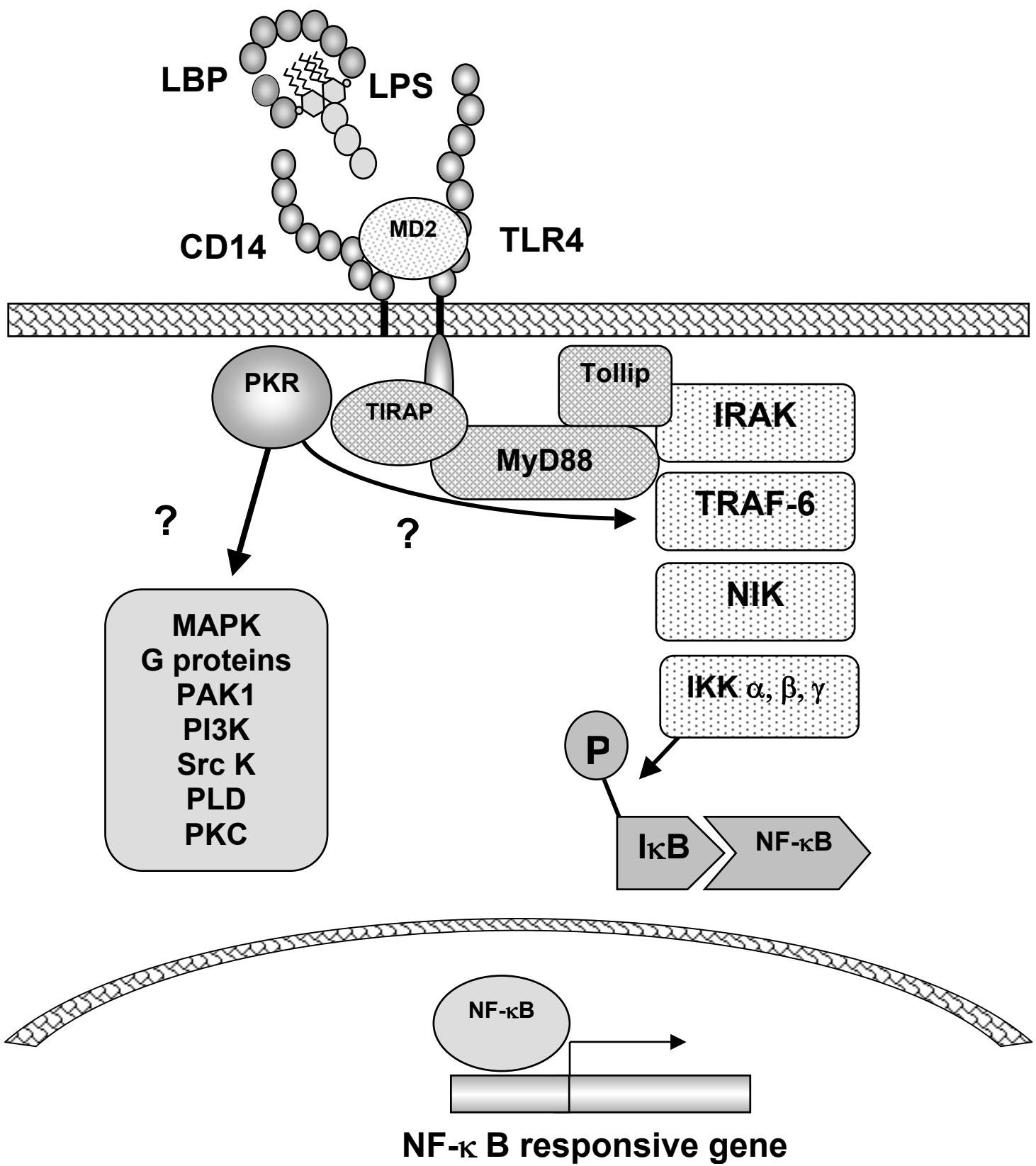
### **LPS Recognition and Signaling**

During infection, free LPS or LPS complexed to outer membrane proteins is released from multiplying and dying Gram negative bacteria (16). Once released into the circulation, LPS has been shown to associate with a broad spectrum of host serum proteins. While most of these proteins serve to neutralize the endotoxic activity of LPS (*e.g.*, high-density lipoprotein (HDL),

bactericidal permeability-increasing protein (BPI), and the cationic proteins: CAP 18, CAP 37, and P15A/P15B), LPS-binding protein (LBP) is instrumental in augmenting the bioactivity of LPS (Figure 2) (17). A role for LBP in cellular LPS recognition and signaling was evident when antibody neutralization of LBP and targeted gene deletion (LBP<sup>-/-</sup>) resulted in decreased production of LPS-induced tumor necrosis factor (TNF)- $\alpha$  and enhanced protection from LPS-induced lethality (18, 19). The mechanism by which LBP enhances LPS bioactivity is attributed to its ability to catalyze the transfer of monomeric LPS from micelles to CD14, an essential component for initiating LPS-induced cellular signal transduction (discussed in more detail below) (20). In addition to augmenting LPS bioactivity, LBP opsonizes LPS-coated particles (*e.g.*, Gram negative bacteria) for delivery to CD14 on the surface of monocytes and neutrophils for subsequent phagocytosis (21). The identification of this role for LBP, in fact, led to the finding that CD14 served as a receptor for LPS (22, 23).

CD14 is a glycosylphosphatidylinositol (GPI)-anchored membrane protein (mCD14) expressed on the surface of phagocytic cells such as monocytes, macrophages, and neutrophils (24, 25). Additionally, CD14 exists as a soluble form (sCD14) in the serum where it mediates LPS-induced cellular responses in cells lacking mCD14 like endothelial cells, epithelial cells, dendritic cells, and fibroblasts (11, 26, 27). Once it was noted that LPS-LBP complexes interacted with CD14, evidence to support the role of CD14 in LPS-induced cellular

**Figure 2.** LPS Signaling. During an infection, LPS is released into the circulation where it associates with LBP. LBP then catalyzes the transfer of monomeric LPS to either soluble or membrane bound CD14 whereupon it associates with TLR4, the primary signal transducer in response to agonist LPS. TLR4, coupled with the accessory protein MD2, is activated and results in the recruitment of the adaptor proteins MyD88, TIRAP, and Tollip. These proteins, in turn, activate a signaling pathway that is initiated by IRAK and culminates in the phosphorylation and subsequent degradation of the inhibitory protein of NF $\kappa$ B, I $\kappa$ B. A more detailed description of the signaling proteins involved in this pathway is provided in the text. Following activation, NF $\kappa$ B then translocates to the nucleus where it serves as a transactivating factor for a broad array of immune response genes. The panel on the left contains a list of signaling proteins that have been implicated in LPS-induced responses, but their association with TLR4 has not been fully elucidated as indicated by the question marks. Abbreviations for all proteins are defined in the text.



responses was provided by numerous studies. Monoclonal antibodies directed against CD14 have been shown to inhibit LPS-induced TNF- $\alpha$  production and protein tyrosine phosphorylation in macrophages (23, 28). Furthermore, transfection of CD14 into various cell types that do not normally express mCD14 either made the cells responsive to LPS or increased the sensitivity of the cells to lower concentrations of LPS (29, 30). Likewise, transgenic mice that overexpress human CD14 were rendered hypersensitive to the effects of LPS (31). Perhaps the most compelling evidence for a critical role for CD14 in LPS signaling was provided with the demonstration that mice with a targeted mutation in CD14 (*i.e.*, CD14 knockout mice) were able to survive a lethal injection with both LPS and *Escherichia coli* (32). In addition, CD14 knockout mice failed to produce the cytokines, TNF- $\alpha$  and interleukin (IL)-6, in response to LPS (32). However, it should be pointed out that other studies have demonstrated that extremely high concentrations of LPS can activate cells in the absence of CD14, supporting the existence of a CD14-independent pathway for LPS activation (23, 33, 34).

While CD14 is clearly involved in mediating cellular responses to physiologic concentrations of LPS, its lack of a transmembrane domain precludes it from functioning as a signal transducer from the exterior to the interior of the cell (35). Consequently, a co-receptor that interacts with CD14 and/or CD14-bound LPS and serves as the true intracellular signaling moiety was sought for nearly a decade after the discovery of CD14. Recently, several investigations have revealed that Toll-like receptor (TLR)4 serves as the primary

transmembrane signal transducer in response to enterobacterial LPS (36-38). The TLRs are a family of closely related transmembrane proteins that transduce intracellular signals to cells in response to a variety of microbial agents (39). The mammalian TLRs are homologous to the *Drosophila* Toll protein, a type I transmembrane protein that is essential for dorsal ventral patterning during embryogenesis and resistance to fungal infection in adult flies (40, 41). The TLRs contain a series of leucine-rich repeat motifs in the extracellular domain, a cysteine-rich region proximal to the membrane, and an intracellular domain with Toll/IL-1 receptor (TIR)-like homology (42). Like the IL-1 receptor, Toll was found to mediate a signaling pathway that parallels the steps required for nuclear translocation of the transacting factor, nuclear factor- $\kappa$ B or NF- $\kappa$ B, in mammalian cells (42). In fact, the first indication that TLR4 may be involved in LPS signaling was noted when transfection of THP-1 monocytes with a constitutively active TLR4 construct resulted in the activation of NF- $\kappa$ B and AP-1, the up-regulation of B7.1 expression, and the production of IL-1 $\beta$ , IL-6, and IL-8 (36). Further evidence was provided when genetic studies of the *Lps<sup>d</sup>* locus of LPS-hyporesponsive C3H/HeJ and C57BL/10ScCR mice revealed a missense and deletion mutation, respectively, in the *tlr4* gene (37, 38). Additional confirmation of the role of TLR4 in LPS signaling was demonstrated by the LPS-hyporesponsive phenotype of TLR4-deficient mice, as well as the inability of TLR4 $^{-/-}$  macrophages to respond to LPS and free lipid A (43). While the involvement of TLR4 in LPS-induced cellular responses is clear, the roles of other

cellular surface proteins that have been associated with LPS-induced cellular responses (*e.g.*, the  $\beta$ 2-integrins, CD11a/CD18, CD11b/CD18, and CD11c/CD18, moesin, and CD55) have yet to be fully elucidated (11).

The initiation of LPS-induced signal transduction not only requires TLR4, but also an accessory protein, MD-2, which binds to the ectodomain of TLR4 (44). The role of MD-2 has not been clearly defined, but it has been proposed that it may either stabilize the formation of putative TLR4 dimers or bind directly to LPS prior to interacting with TLR4 (45). Following LBP- and CD14-mediated interactions with LPS, the TLR4-MD-2 complex is activated through an unknown mechanism to initiate intracellular signaling pathways that closely parallel those of the IL-1R family (46). While it is not entirely clear whether ligand-activated TLR4 undergoes dimerization or multimerization like members of the IL-1R family, the subsequent recruitment of a primary adaptor protein, myeloid differentiation factor 88 (MyD88), has been demonstrated to be shared by both receptor families (46, 47). Upon TLR4 activation, the carboxy (C)-terminal TIR domain of MyD88 associates with the cytoplasmic TIR domain of TLR4 (47). The direct interaction of MyD88 with TLR4 was demonstrated through two-hybrid analysis and co-immunoprecipitation studies with full-length TLR4, but not TLR4 lacking the cytoplasmic TIR domain (47, 48). The essential role of MyD88 in LPS-induced signaling was clarified by the demonstration that MyD88-deficient mice and macrophages fail to produce TNF- $\alpha$  and IL-6 in response to LPS stimulation and that MyD88 knockout mice were refractory to LPS-induced

lethality (49). Despite the abrogation of cytokine production, the MyD88-deficient macrophages were capable of delayed mitogen activated protein (MAP) kinase and NF- $\kappa$ B activation, suggesting the existence of a MyD88-independent pathway (49). Recently, the discovery of a second adaptor protein for TLR4, TIR domain containing adaptor protein (TIRAP) or MyD88-adaptor-like protein (Mal), revealed an alternate mechanism whereby signaling could still occur in the absence of MyD88, perhaps accounting for the residual, delayed NF- $\kappa$ B and MAPK activation observed in macrophages derived from MyD88 knockout mice (50, 51). TIRAP/Mal appears to bind to a site on TLR4 distinct from MyD88, and the work of Fitzgerald et al. indicates that TIRAP/Mal also interacts with MyD88 (51). Blocking TIRAP/Mal blocks both MyD88-dependent and MyD88-independent TLR4 signaling, but fails to block signaling mediated by TLR2, TLR9, IL-1, or IL-18 receptors. This suggests that TIRAP/Mal is preferentially associated with TLR4. The interaction of MyD88 with TLR4, in turn, leads to the association and activation of IL-1 receptor associated kinases (IRAK) family members (*e.g.*, IRAK-1, IRAK-2, and IRAK-M) via the amino (N)-terminal death domains of both IRAK and MyD88 (52). Interestingly, there is recent evidence to suggest that while MyD88 associates preferentially with IRAK-1 and IRAK-2, TIRAP/Mal is found in association with IRAK-2 only (51). Following its dissociation from the receptor complex, phosphorylated IRAK associates with a TNF receptor-associated factor (TRAF) 6, which in turn, results in the phosphorylation and activation of TRAF6 (53).

The multivalent adapter or scaffolding protein, TRAF6, occupies a central position upstream of the NF- $\kappa$ B-inducing pathway and the major MAP kinase pathways (11, 42, 45). Among the signaling intermediates that have been shown to associate with TRAF6 are members of the MAP kinase kinase kinase (MAP3K) family {e.g., transforming growth factor- $\beta$ -activated kinase-1 (TAK1), MAPK/ERK kinase kinase I (MEKK1), MEKK2, and MEKK3}, the MAP3K-like kinase, NF- $\kappa$ B inducing kinase (NIK), and atypical members of the protein kinase C (PKC) family (11). The association of TAK1, and a specific activator, TAK1 binding protein 1 or 2 (TAB1/2), with TRAF6 results in the activation of NIK, as well as the c-Jun N-terminal kinase/stress-activated protein kinase 1 (JNK/SAPK1) and p38/SAPK2 pathways (54, 55). Activated NIK, in association with other MAP3K family members, then activates I- $\kappa$ B kinase (IKK) complexes in the cytoplasm (56). Once the IKKs phosphorylate the inhibitory protein of NF- $\kappa$ B (I- $\kappa$ B), I- $\kappa$ B is ubiquitinylated and degraded and the transcription factor, NF- $\kappa$ B, is released and translocated to the nucleus (57). Another intermediate signaling protein, ECSIT (evolutionarily conserved intermediate in the Toll/IL-1 signal transduction pathway), bridges the interaction of TRAF6 with MEKK1, which has been shown to mediate the activation of the JNK/SAPK1 pathway and IKK complexes (58). Direct evidence to support the roles of TRAF6, TAK1, NIK, and ECSIT in TLR4-mediated signaling was provided by studies in which the dominant-negative forms of these proteins inhibited the activation of downstream signaling intermediates and/or transactivation factors (42). In

addition to the intracellular signaling intermediates that have been demonstrated to associate with the TLR4-signaling pathway, there remain several others that have been implicated in LPS-induced responses that have not yet been completely linked to TLR4 (*e.g.*, phosphatidylinositol 3-kinases (PI3K), phospholipase D (PLD), p21-activated kinase 1 (PAK), monomeric and heterotrimeric G proteins, and src family tyrosine kinases) (11). The culmination of all LPS-induced intracellular signaling is the rapid transactivation of multiple immune and inflammatory response genes by NF- $\kappa$ B, as well as numerous members of the AP-1, Ets, EGR, C-EBP, and ATF/CREB families of transcription factors (59).

### **The Inflammatory Response to LPS**

The ability of a host to recognize a pathogenic organism and mount an appropriate response to control infection is essential for host survival. Unlike the adaptive immune system which requires immunological memory to recognize a pathogenic organism, the innate immune system utilizes pattern recognition receptors (PRRs) like the TLR family to recognize invariant molecular structures or "pathogen-associated molecular patterns" (PAMPs) that are representative of large groups of pathogenic organisms. The highly conserved lipid A region of LPS represents a PAMP that is indicative of a Gram negative infection. TLR4 serves as a key defense mechanism in the innate immune response to Gram negative infection in its capacity to mediate the recognition of LPS, and

subsequently, elicit the signaling pathways that direct the inflammatory response. The inflammatory response to LPS involves the release of a broad spectrum of endogenous mediators like the cytokines TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, IL-12, IL-18, and colony stimulating factors (CSFs); the lipid mediators platelet activating factor, prostaglandin E<sub>2</sub>, thromboxane A<sub>2</sub>, and leukotriene C<sub>4</sub>; and the reactive oxygen intermediates superoxide anion, hydroxyl radicals, and nitric oxide; as well as the activation of the complement pathway (11, 60, 61). The primary release of these endogenous mediators serves to activate multiple cell types and physiologic systems, particularly circulation and coagulation, which in turn, initiate a secondary series of reactions to further amplify the inflammatory cascade. While the rapid activation of inflammatory mediators in response to LPS is beneficial for the elimination of microbial pathogens, the dysregulated overproduction of these inflammatory mediators leads to the toxicity associated with sepsis and septic shock.

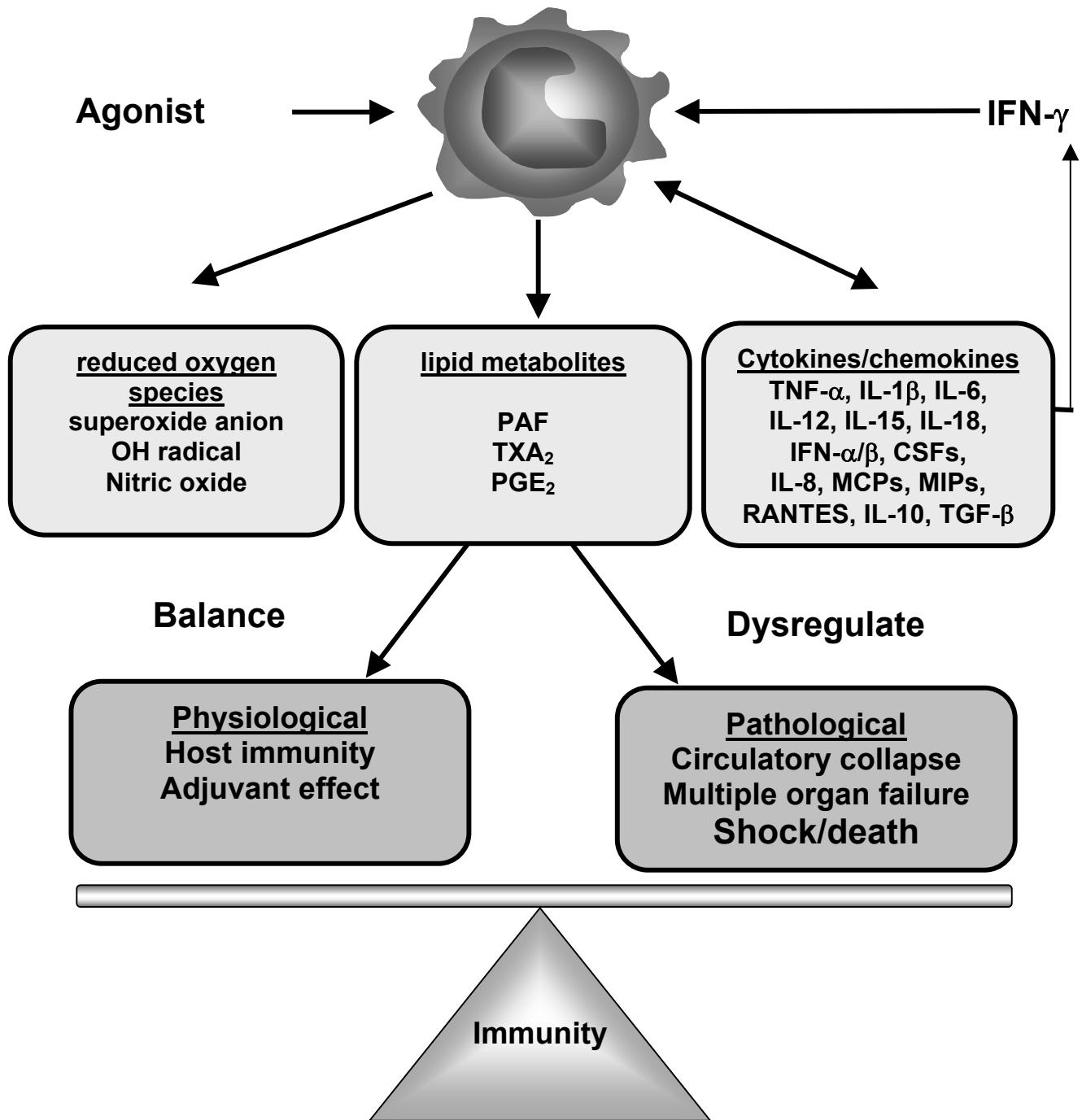
Although LPS is capable of activating several cell types, the principal mediator of LPS-induced responses is the macrophage (Figure 3). In support of their central role is the observation that macrophages exposed to LPS *in vitro* produce most of the inflammatory cytokines (*e.g.*, TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-12, IL-18, etc.) and bioactive mediators (*e.g.*, nitric oxide, reactive oxygen intermediates, and arachidonic acid metabolites) that are detected *in vivo* upon LPS administration (61, 60). Further support was demonstrated when LPS-hyporesponsive mice (*lps<sup>d</sup>*) were rendered responsive to LPS following the

adoptive transfer of either macrophage progenitors or mature macrophages derived from LPS-responsive mice (62, 63). Additionally, B-cell deficient (*CBA/N xid*) mice, T-cell deficient (*nu/nu*) mice, severe combined immunodeficient (SCID) mice, and splenectomized mice exhibited normal responsiveness to LPS, suggesting that cells of the lymphoid system are not required for mediating the effects of LPS (64-67). More recently, macrophages were revealed to be the primary cellular source of IL-1 $\beta$ , IL-6, IL-10, IL-12p40, and TNF- $\alpha$  mRNA in the liver, and IL-1 $\beta$ , IL-6, and IL-12p40 mRNA in the spleen of macrophage-depleted mice after administration of LPS (68).

One of the hallmarks of exposure to LPS is the rapid and coordinated recruitment of leukocytes into host tissues, particularly the lung and liver. While the influx and activation of leukocytes is essential to innate host defense against microbial pathogens, the accumulation of leukocytes, in combination with their overproduction of proinflammatory mediators, are believed to result in the tissue damage that precedes multiple organ failure. Within one hour of LPS exposure, the number of circulating neutrophils drops precipitously by 50 to 75% as a result of margination and aggregation of neutrophils in the microvasculature (69). Almost 8-12 hours after LPS exposure, there is a dramatic increase in newly circulating neutrophils accompanied by a decrease in peripheral monocytes and lymphocytes (69). In concordance with changes in circulating leukocytes, numerous investigations in humans as well as rodent models of endotoxemia and sepsis have observed that neutrophils predominate during early tissue

**Figure 3.** Physiological Consequences of Agonist LPS-Macrophage Interactions.

While LPS does exert its effects on diverse cell types, it is the interaction with macrophages that is pivotal in mediating the inflammatory response to LPS. The interaction of agonist LPS with macrophages triggers the release of several inflammatory mediators to include: reduced oxygen species, lipid metabolites, cytokines, and chemokines. These mediators then exert their effects on diverse cell types and some act back on macrophages in an autocrine loop to result in the propagation of an inflammatory cascade that escalates rapidly. During limited or local exposure to LPS, this process is beneficial to the host as it results in augmented host resistance. However, overwhelming systemic exposure to LPS results in a dysregulation of the inflammatory cascade that ultimately culminates in the pathophysiology associated with endotoxemia.



infiltration, followed by a later accumulation of monocytes and lymphocytes (60, 70-72). Leukocyte recruitment into extravascular sites requires a complex orchestration of adhesion molecule expression on leukocytes and endothelial cells, in addition to, the establishment of a chemotactic gradient with chemotactic factors such as chemokines (73) (described in more detail below in "Chemokines in Leukocyte Trafficking"). Inflammatory mediators like TNF- $\alpha$  and IL-1 $\beta$  are instrumental in this process due to their ability to up-regulate cell-surface adhesion molecule expression, enhance vascular permeability for transmigration of cells, and further induce chemokine expression (61). The tissue injury that occurs in the presence of activated leukocytes is attributable to the production of proteases, lysosomal enzymes, and reactive oxygen species (74). In severe cases of endothelial cell damage and tissue injury, vascular collapse ensues along with widespread ischemia, hypoxia, organ failure, and ultimately death (75). Studies involving antibody neutralization of chemokines and adhesion molecules, chemokine and adhesion molecule knockout mice, and neutrophil-depleted mice have demonstrated that disruption of leukocyte recruitment into target organs resulted in protection from organ injury and/or LPS-induced lethality (76-79). Clearly, the influx of activated leukocytes into host tissues contributes enormously to the pathogenesis associated with LPS. In recent years, chemokines have emerged as some of the most potent mediators of leukocyte migration and activation, and consequently, play a pivotal role in the inflammatory response to LPS.

## Properties of the Chemokine Superfamily

After the breakthrough discovery and cloning of the prototypical chemokine, IL-8 (80-82), the identification and characterization of a large superfamily of structurally and functionally related cytokines rapidly ensued {reviewed in 83-87}. These proteins have been collectively termed "chemokines" (*chemotactic cytokines* ), since they were originally characterized as secreted proteins that shared the capacity to induce leukocyte migration (88, 89). In contrast with classical chemoattractants (e.g., complement component C5a, formylated peptides, and arachidonic acid metabolites PGE<sub>2</sub> and LTB<sub>4</sub>), chemokines are more selective in their ability to recruit specific cell populations. Chemokines are produced by virtually every cell type in the body in response to pro-inflammatory mediators, infectious pathogens, and pathologic disease states, as well as during normal homeostasis. While the induction of leukocyte migration remains the unifying biological role of chemokines, extensive investigations have uncovered diverse ancillary roles. To date, over 50 unique chemokines have been identified. Despite its large size, the chemokine superfamily is a surprisingly homogeneous family of proteins that share numerous characteristic features.

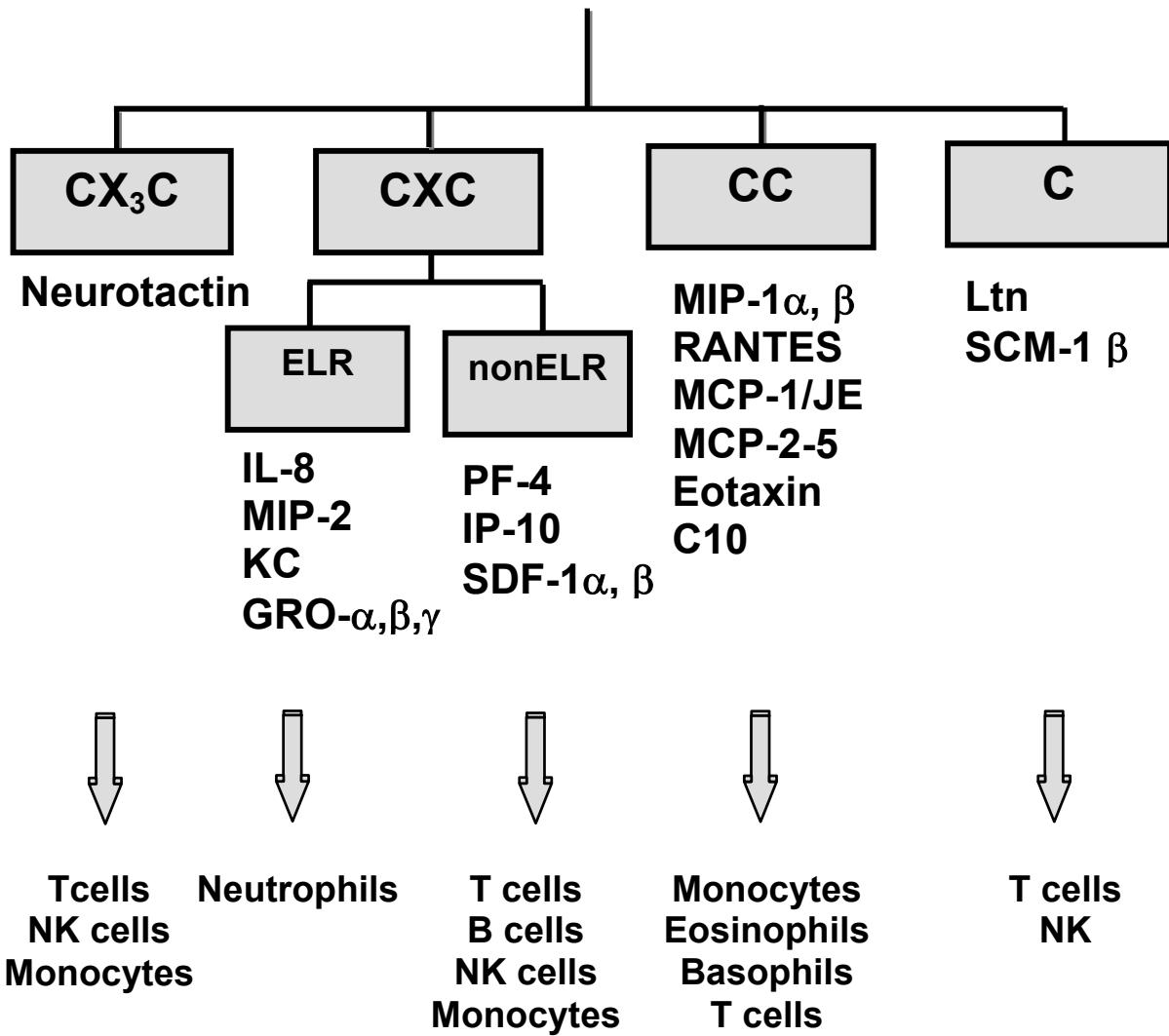
Chemokines are small (7-16 kDa), basic proteins that exhibit significant amino acid sequence homology. One hallmark of proteins in the chemokine superfamily is four positionally conserved cysteine residues. Based on the arrangement of the first two cysteine residues within the N-terminus, the

chemokine superfamily is categorized into four subfamilies, CXC ( $\alpha$ ), CC ( $\beta$ ), C ( $\gamma$ ), and CX<sub>3</sub>C ( $\delta$ ) (Figure 4). The formation of two disulfide bonds between the 1<sup>st</sup> and 3<sup>rd</sup> and 2<sup>nd</sup> and 4<sup>th</sup> cysteine residues confers the three-dimensional structure essential for biological activity of the chemokines. The C chemokines, lymphotactin and single cysteine motif (SCM)-1 $\beta$ , are the only exceptions since they each have just two cysteine residues and, consequently, a single disulfide bond (90, 91).

Considering their substantial amino acid homology, it is not surprising that structural analysis of various chemokines has revealed several characteristic features (92-97). Preceding the first cysteine is a short, N-terminal domain that contributes to the selectivity of receptor binding. For example, in the CXC subfamily, the presence of a glutamate-leucine-arginine (ELR) motif immediately preceding the CXC signature is the primary determinant for the ability of these chemokines to recruit neutrophils (98). Not only did deletion of this ELR motif in IL-8 abrogate its ability to recruit neutrophils, but the insertion of this motif into non-ELR-containing platelet factor 4 (PF-4) enabled it to bind and activate neutrophils (98, 99). While an analogous motif has not been identified in CC chemokines, the presence of an intact N-terminus and specific amino acid residues is critical for biological activity (100, 101). Another characteristic feature of all chemokines is a backbone of 3 anti-parallel  $\beta$ -sheets that rests between the connecting loops formed by the disulfide bonds. Mutational analysis of this region has established that amino acids whose side chains extend into the cleft

**Figure 4.** The Chemokine Superfamily. Chemokines are small (7-16 kDa), secreted proteins that share the capacity to induce leukocyte migration. The chemokine superfamily is comprised of over 50 members which are divided into four subfamilies depending on the arrangement of the positionally conserved cysteine motif in the N-terminus: CX3C, CXC, CC, and C. In the CXC subfamily, the presence of a glutamate-leucine-arginine (ELR) motif immediately preceding the CXC signature is the primary determinant for the ability of these chemokines to recruit neutrophils (98). Traditionally, chemokine subfamilies were distinguished on the basis of their leukocyte specificity, namely that CXC chemokines induce the directional migration of neutrophils and CC chemokines exert their effects on monocytes and lymphocytes. With the discovery of new chemokines and more thorough investigation, this distinction is no longer valid as indicated by the primary cell types listed beneath each chemokine subfamily.

## The Chemokine Superfamily



between the  $\beta$ -sheets and the C-terminal  $\alpha$ -helix are critical for functional activity (101, 102). The C-terminus is comprised of an  $\alpha$ -helix of 20-30 amino acids that binds heparin-bearing proteoglycans on vascular endothelium or extracellular matrix proteins in the tissue (103, 104). The immobilization of chemokines through the low affinity binding of the C-terminus allows for the establishment of a solid-phase, or haptotactic, gradient and facilitates effective presentation to rolling leukocytes at the site of inflammation.

In addition to structural similarities, mapping studies have determined that subfamily genes are clustered at specific chromosomal loci. With few exceptions, the genes encoding CXC chemokines map to human chromosome 4q12-21 (mouse chromosome 5), the CC chemokine genes map to human chromosome 17q11.2-12 (mouse chromosome 11), and the C chemokine genes map to human and mouse chromosome 1 (83, 91, 105-107). Chromosomal clustering cannot be assessed for the CX<sub>3</sub>C subfamily because it presently contains only one member, fractalkine (human chromosome 16; mouse chromosome 8) (108, 109). Besides chromosomal localization, the gene structure within a subfamily is also highly conserved (105-107). For instance, the majority of CXC genes contain 4 exons and 3 introns, whereas the CC genes contain 3 exons and 2 introns. In both subfamilies, the first intron separates the leader sequence from the mature protein. Furthermore, the messenger RNA (mRNA) for both subfamilies contains conserved single open reading frames, signal sequences in the 5' region, and AT rich sequences in the 3' untranslated region

(UTR). Typically, chemokine mRNAs encode proteins ranging in size from 90-150 amino acids, of which 20-25 amino acids comprise the leader sequence that is necessary for secretion. One notable exception is fractalkine, which shares a chemokine-like domain at its N-terminus, but also has an additional mucin-coated stalk attached to a cytoplasmic domain (108, 109). The co-clustering of subfamily genes and conserved sequence structure suggests that chemokines may have arisen from a common ancestral gene that underwent gene duplication and subsequent divergence.

### **Chemokine Subfamilies**

Of the four subfamilies, CXC ( $\alpha$ ), CC ( $\beta$ ), C ( $\gamma$ ), and CX<sub>3</sub>C ( $\delta$ ), the CXC and CC contain the majority of the known chemokines and have been the most extensively studied to date. Traditionally, the two subfamilies were distinguished on the basis of their leukocyte-specificity, namely that CXC chemokines induced activation and directional migration of neutrophils, while CC chemokines exerted their effects predominantly on monocytes and lymphocytes (105). With the discovery of new chemokines and more thorough investigation, this distinction is no longer valid (discussed in more detail below). In the remaining C and CX<sub>3</sub>C subfamilies, a limited number of chemokines have been identified for each subfamily. The C chemokines, lymphotactin (SCM-1 $\alpha$ ) and SCM-1 $\beta$ , are produced primarily by activated CD8 $^{+}$  T cells and are active on CD4 $^{+}$  T cells and NK cells (90, 91). The CX<sub>3</sub>C chemokine, fractalkine (mouse

neurotactin), is the only known membrane-bound chemokine and serves as a CD4<sup>+</sup> T cell, NK cell, and monocyte chemoattractant (108, 109). Because of the breadth of the chemokine superfamily, only those chemokines of historical relevance or those that were investigated for purposes of this dissertation work (*i.e.*, KC, MIP-2, IP-10, JE/MCP-1, MCP-5, MIP-1 $\alpha$ , MIP-1 $\beta$  and RANTES) will be discussed below.

The CXC or  $\alpha$  subfamily is further divided into ELR<sup>+</sup> and ELR<sup>-</sup> chemokines. As previously discussed, the presence of the ELR motif in the N-terminus distinguishes those chemokines that recruit neutrophils (ELR<sup>+</sup>) from those that are inactive toward neutrophils (ELR<sup>-</sup>). Several ELR<sup>+</sup> CXC chemokines have been identified in humans: IL-8, granulocyte chemoattractant protein-2 (GCP-2), growth related oncogene/melanoma growth-stimulatory activity (GRO/MGSA- $\alpha/\beta/\gamma$ ); neutrophil-activating peptide-2 (NAP-2); and epithelial cell-derived neutrophil attractant-78 amino acid (ENA-78) (85). The most potent neutrophil chemoattractant, IL-8, is the prototypical ELR<sup>+</sup> CXC chemokine that was originally isolated from the supernatants of LPS-stimulated human blood mononuclear leukocytes (80). IL-8 is produced by numerous cell types such as monocytes, neutrophils, fibroblasts, endothelial cells, epithelial cells, mast cells, and keratinocytes (83). Although primarily a neutrophil chemoattractant, IL-8 has been shown to induce chemotaxis in basophils, monocytes, NK cells, endothelial cells, and a minor subset of T lymphocytes (110). Besides its chemotactic properties, IL-8 up-regulates surface expression of  $\beta_2$  integrins,

induces L-selectin shedding from the surface of neutrophils, promotes generation of superoxide and hydrogen peroxide, and induces the release of myeloperoxidase, arachidonic acid metabolites, and matrix metalloproteinases (*i.e.*, gelatinase-B,  $\beta$ -glucuronidase, and elastase) (110). In concordance with its ability to activate neutrophil chemotaxis *in vitro*, IL-8 has been shown to function as a neutrophil chemoattractant *in vivo* as well. In human and animal studies, intradermal and subcutaneous injections of IL-8 resulted in a rapid and concentration-dependent recruitment of neutrophils to the local site of administration (111, 112). Likewise, elevated levels of IL-8 mRNA and/or protein have been recovered from inflammatory sites of pathologic disease states that are characterized by an influx of neutrophils (*e.g.*, inflammatory bowel syndrome, gingivitis, acute respiratory distress syndrome, sepsis/endotoxemia, etc.) (86). IL-8 binds with high affinity to two chemokine receptors, CXCR1 and CXCR2 (113, 114). CXCR1 selectively binds IL-8 and GCP-2, whereas CXCR2 is shared by all ELR<sup>+</sup> CXC chemokines (85).

To date, the murine homologues of human IL-8 and CXCR1 have not been identified. However, the predominant inflammatory ELR<sup>+</sup> CXC chemokines in mice, KC and macrophage inflammatory protein (MIP)-2, have been described as the functional homologues of human IL-8. Originally isolated from platelet-derived growth factor (PDGF)-induced BALB/c 3T3 fibroblasts, KC is a neutrophil chemoattractant that is produced by multiple cell types (*e.g.*, macrophages, fibroblasts, and endothelial cells) and shares 65% amino acid

sequence identity with human GRO- $\alpha$  (115-117). MIP-2, first isolated from LPS-stimulated RAW 264.7 macrophages, is another neutrophil chemoattractant that is primarily produced by macrophages and shares 60% amino acid sequence identity with human GRO- $\beta$  and GRO- $\gamma$  (118, 119). Like IL-8, KC and MIP-2 induce neutrophil degranulation and up-regulation of  $\beta_2$  integrin expression, but only KC promotes respiratory burst in neutrophils (118, 120). KC and MIP-2, along with other known murine ELR<sup>+</sup> CXC chemokines (*e.g.*, LPS-induced CXC chemokine (LIX), ENA-78, and GCP-2), bind with high affinity to the murine homologue of CXCR2 (120-122). While CXCR2 is the only known receptor for ELR<sup>+</sup> CXC chemokines in mice, neutrophil recruitment can also be mediated by the CC chemokine, MIP-1 $\alpha$ , and its cognate receptor, CCR1, on neutrophils (71, 123).

The presence or absence of the ELR motif also distinguishes between angiogenic and anti-angiogenic properties, respectively. The biological process of blood vessel growth, or angiogenesis, and endothelial cell chemotaxis are stimulated by ELR<sup>+</sup> CXC chemokines like IL-8, GRO, and ENA-78 (84, 124). The increased expression of ELR<sup>+</sup> CXC chemokines, most notably IL-8, in experimental models and pathologic disease states characterized by neovascularization (*i.e.*, development, wound healing, and tumorigenesis) further supports their involvement in angiogenesis (84, 124). Alternatively, ELR- CXC chemokines like interferon- $\gamma$ -inducible protein-10 (IP-10), monokine inducible by interferon- $\gamma$  (MIG), interferon-inducible T-cell alpha chemoattractant

(I-TAC), and platelet factor 4 (PF4), have potent anti-angiogenic properties (125). In addition to their capacity to inhibit endothelial cell proliferation and migration *in vitro*, ELR<sup>-</sup> CXC chemokines have been shown to inhibit wound healing, reduce chronic inflammatory diseases, and attenuate tumor growth and metastasis *in vivo* (84, 124). IFN- $\gamma$  figures prominently as a regulatory cytokine to promote anti-angiogenesis. Not only does IFN- $\gamma$  induce the expression of IP-10, MIG, I-TAC, and PF4 and their receptor, CXCR3, but it also inhibits the expression of angiogenic ELR<sup>+</sup> CXC chemokines like IL-8, GRO, and ENA-78 (126-133).

In addition to their anti-angiogenic effect on endothelial cells, the ELR<sup>-</sup> CXC chemokines also demonstrate functional diversity as chemoattractants. The closely related ELR<sup>-</sup> CXC chemokines, IP-10, MIG, and I-TAC, selectively recruit activated T cells and have no effect on resting or naïve T cells (134-136). Only IP-10 has demonstrated the ability to recruit monocytes and NK cells as well (128, 137). Although all three ligands bind to CXCR3, I-TAC is proposed to be the dominant ligand because it has the highest affinity and potency for CXCR3 (128). Their inducibility by IFN- $\gamma$  suggests that all three chemokines may be preferentially involved in Th1 immune responses. In fact, the most well-characterized, IP-10, has been found to be expressed in classic Th1 immune responses such as delayed-type hypersensitivity reactions, tuberculoid leprosy, and psoriatic plaques (138, 139). While IP-10 and I-TAC are highly inducible

genes, both are expressed in normal tissue like thymus, spleen, and pancreas, where they may be involved in the trafficking of activated T cells (128).

With over 27 identified members, the CC or  $\beta$  subfamily also exhibits a diverse range of target cell specificities and functions. The prototype CC chemokine, monocyte chemoattractant protein (MCP)-1, was originally isolated from non-human primate smooth muscle cells as a monocyte chemoattractant that had no effect on neutrophils (140). Only after human MCP-1 was cloned and sequenced was it realized that the murine homologue, JE, was actually identified years earlier with KC from the supernatants of PDGF-stimulated 3T3 fibroblasts (115, 141-144). In addition to being a potent monocyte chemoattractant, MCP-1 has been shown to recruit memory T cells, NK cells, basophils, mast cells, and immature dendritic cells (145). *In vitro* studies with monocytes have revealed that MCP-1 up-regulates  $\beta_2$  integrin expression, promotes the release of lysosomal enzymes, induces IL-1, IL-4 and IL-6 production, and enhances the tumoricidal activity of monocytes (141, 146). While MCP-1 is a weak chemoattractant of basophils and mast cells, its ability to induce histamine and leukotriene C<sub>4</sub> release in these cell types is almost as robust as IgE-mediated stimulation (147). Several *in vivo* models, most notably MCP-1-transgenic and -deficient mice, have validated the role of MCP-1 as a predominantly monocytic chemoattractant (148-152). More recently, a study with MCP-1-deficient mice has demonstrated a role for MCP-1 in the direct control of Th2 polarization (153). While trafficking of naïve T cells was

undisturbed, MCP-1<sup>-/-</sup> mice were unable to mount a Th2 immune response due to their inability to produce IL-4, IL-5, or IL-10, and consequently, initiate immuno-globulin E (IgE) class switching (153). Additionally, MCP-1 has been associated with pathologic processes with a chronic inflammatory component such as atherosclerosis, rheumatoid arthritis, glomerulonephritis, chronic allergic/asthmatic inflammation, and pulmonary fibrosis (86). However, its role in inflammation is not clear as antibody neutralization has indicated a protective role for MCP-1 in lethal endotoxemia (154). Unlike the promiscuity exhibited by most chemokines, MCP-1 binds only one receptor, CCR2, which has two isoforms in humans (CCR2a and CCR2b) and one in mice (155, 156).

Another closely related CC chemokine, MCP-5, was cloned by two independent groups by exploiting the highly conserved sequence homology of the chemokines (157, 158). MCP-5 exhibits similar chemotactic properties as MCP-1 on monocytes, T lymphocytes, and basophils, but is generally less potent than MCP-1 (145). To date, MCP-5 has been identified only in the mouse, and like murine JE, it is believed to be another functional homologue of human MCP-1 (158). *In vivo*, the expression of MCP-5 has been demonstrated in murine models of peritoneal sepsis and allergic inflammation (157, 159, 160). Studies involving antibody neutralization of MCP-5 have shown significant reductions in the number of monocytes, lymphocytes, and eosinophils infiltrating the lung during allergic inflammation (157, 160). Just as human MCP-1 binds only to CCR2, both murine JE and MCP-5 bind only to CCR2 (158).

Among the other well-characterized CC chemokines are the macrophage inflammatory proteins (MIPs), MIP-1 $\alpha$  and MIP-1 $\beta$ . Since their initial co-purification from supernatants of LPS-activated murine macrophage cell lines (RAW 264.7), they have been reported to be produced by a variety of cell types (*i.e.*, lymphocytes, dendritic cells, NK cells, neutrophils, mast cells, endothelial cells, and fibroblasts) (70, 161, 162). While MIP-1 $\alpha$  and MIP-1 $\beta$  share 70% amino acid sequence homology and overlapping biological functions, they are distinct proteins with unique physiological roles. For instance, MIP-1 $\alpha$  and MIP-1 $\beta$  both have the ability to induce chemotaxis in monocytes, T cells, B cells, NK cells, and eosinophils, but only MIP-1 $\alpha$  is capable of inducing chemotaxis in dendritic cells (83). Additionally, MIP-1 $\alpha$  is not only more potent than MIP-1 $\beta$  in attracting monocytes, but also, is capable of inducing monocytes/macrophages to produce TNF- $\alpha$ , IL-1 $\beta$ , and IL-6, to enhance tumoricidal activity, and to up-regulate integrin expression (163, 164). Another noted difference is that MIP-1 $\alpha$  predominantly recruits CD8 $^{+}$  T cells, while MIP-1 $\beta$  preferentially induces CD4 $^{+}$  T cell recruitment (165, 166). While MIP-1 $\alpha$  functions as a potent inhibitor of hematopoietic stem cell proliferation, MIP-1 $\beta$  acts as an antagonist of MIP-1 $\alpha$ -induced stem cell proliferation (167). Neither MIP-1 $\alpha$  nor MIP-1 $\beta$  have been shown to induce the migration of neutrophils *in vitro*, yet the MIPs were originally characterized as neutrophil chemoattractants after injection in the footpads of mice resulted in an immediate influx of neutrophils followed by monocytes (168). Antibody neutralization studies have further corroborated the

involvement of MIP-1 $\alpha$  in mediating neutrophil recruitment in animal models of inflammation (71, 169). Of greater significance is their central role as monocyte and lymphocyte chemoattractants as demonstrated by numerous animal models involving antibody neutralization and targeted gene deletion approaches (76). Their overlapping biological functions are not surprising as they both share at least two common receptors: CCR5 and CCR9 (85, 170). The fact that MIP-1 $\alpha$  also binds to CCR1 and CCR4 and MIP-1 $\beta$  binds to CCR8, likely contributes to their unique biological roles (85, 170).

The CC chemokine, RANTES (regulated upon activation normal T cell expressed and secreted), was originally isolated as a transcript present in T cells, but not in B cells (171). While RANTES production also has been demonstrated in monocytes/macrophages, endothelial cells, and fibroblasts, its production in platelets is particularly noteworthy, as it is the only CC chemokine produced by platelets (83, 107). RANTES is a chemoattractant for monocytes, memory T cells, NK cells, eosinophils, and basophils (83). In addition to its chemoattractant properties, RANTES is capable of inducing histamine release in basophils and eosinophils, as well as up-regulating CD11b/CD18 in eosinophils (172). *In vivo*, antibody neutralization studies have demonstrated the contribution of RANTES in recruiting monocytes into the lung during endotoxemia, while in allergic inflammation, RANTES is primarily responsible for the recruitment of eosinophils and lymphocytes into the lung (160, 173). RANTES mediates its effects by binding to several receptors: CCR1, CCR3, CCR4, and CCR5 (162).

Because CCR5 serves as a coreceptor with CD4 for M-tropic strains of HIV-1, the natural ligands of CCR5 (*i.e.*, MIP-1 $\alpha$ , MIP-1 $\beta$ , and RANTES), all serve to block HIV infection in macrophages (174-178).

### **Chemokine Receptors**

Chemokine receptors belong to a superfamily of seven-transmembrane, guanine nucleotide-binding (G) protein-coupled receptors (GPCR). The chemokine receptors are named on the basis of the chemokine subfamilies that they bind: "CXCR1 - 6" bind CXC chemokines, "CCR1 - 11" bind CC chemokines, "XCR1" binds the C chemokines, and "CX<sub>3</sub>CR1" binds the CX<sub>3</sub>C chemokine, fractalkine (84, 179). Like the chemokine subfamilies, most of the chemokine receptor gene subfamilies are clustered within specific chromosomal loci. For example, the genes encoding CXCR1, CXCR2, and CXCR4 are co-localized on human chromosome 2 (mouse chromosome 1, except for CXCR1), and the genes encoding CCR1, CCR2, CCR3, and CCR5 are co-localized on human chromosome 3 (mouse chromosome 9) (87, 180). According to a proposed structural model based on the known structure of rhodopsin, chemokine receptors have an extracellular N-terminus, seven  $\alpha$ -helical plasma membrane-spanning domains, and an intracellular C-terminus (180, 181). Four highly conserved cysteines are predicted to form two disulfide bonds between the 1<sup>st</sup> and 2<sup>nd</sup> extracellular loops and the N-terminus and 3<sup>rd</sup> extracellular loops (182). Site-directed mutagenesis indicates that these cysteines are necessary for

structural stability and ligand binding (183-185). The highly conserved DRYLAIVHA-motif in the second intracellular loop and a methionine residue in the third intracellular loop are known to confer efficient coupling to G proteins, and thus, are critical for signal transduction (183, 186, 187). Like all GPCR, there are numerous serine and threonine residues in the C-terminus which serve as phosphorylation sites for GPCR kinases (188).

Chemokine-receptor interactions are described as promiscuous due to the ability of an individual chemokine receptor to bind multiple chemokines, as well as the ability of an individual chemokine to bind more than one receptor. For instance, chemokine receptors like CXCR2 can bind to IL-8, GRO- $\alpha/\beta/\gamma$ , ENA-78, GCP-2, and NAP-2; and CCR3 can bind to eotaxin, RANTES, MCP-2, MCP-3, and MCP-4 (170). Likewise, chemokines like IL-8 can bind to CXCR1 and CXCR2; and MCP-1 can bind to CCR1-3, CCR5, and CCR9 (189). Although promiscuous, chemokine-receptor interactions are restricted to their respective subfamilies. In other words, CXC chemokines bind to CXC receptors and CC chemokines bind to CC receptors. One exception is the murine CC chemokine, 6Ckine/SLC, which has been shown to bind the CXC chemokine receptor, CXCR3 (130). Besides IL-8, CXC chemokines generally have a high affinity for one receptor, whereas CC chemokines have high affinities for multiple receptors with diverse cellular distributions. Consequently, CXC chemokine-receptor interactions may induce more selective leukocyte responses than CC chemokines.

The most notable example of receptor promiscuity is the Duffy antigen receptor for chemokines (DARC), which binds multiple chemokines in both the CXC (*e.g.*, IL-8, KC, MIP-2, GRO $\alpha$ , NAP-2, ENA-78) and CC (*e.g.*, MCP-1, MCP-3, RANTES) subfamilies (190). Like other chemokine receptors, DARC is comprised of seven transmembrane domains and shares approximately 20% amino acid identity, yet ligand binding does not induce signaling (191-193). Commonly known as the Duffy blood group antigen, DARC serves as a binding protein for the human malarial parasite, *Plasmodium vivax*, on the surface of erythrocytes (192). Besides erythrocytes, DARC is expressed on endothelial cells of postcapillary venules and splenic sinusoids, Purkinje cells in the cerebellum, and limited populations of B and T lymphocytes (192, 194). The biological significance of this receptor has been questionable due to its inability to transduce signals, as well as the apparent "normal" phenotype of Duffy blood group antigen negative individuals. However, the expression of DARC was confirmed on the endothelium of Duffy negative individuals, suggesting a more significant role (194). While its function remains to be elucidated, it has been speculated that it may function as a "biological sink" to remove excess chemokines from the circulation (195). Recently, evidence to support this hypothesis was provided when significantly higher inflammatory cell infiltrates were detected in lung and liver of DARC knockout mice after administration of LPS (196).

## Chemokine Receptor Signaling

Even before the cloning and characterization of the first chemokine receptor, the observation that *Bordetella pertussis* toxin inhibited cellular responses to IL-8 led to the hypothesis that its receptor was coupled to heterotrimeric G $\alpha_i$  proteins (197). The central role of G $\alpha_i$  proteins in chemokine receptor signaling is underscored by the complete inhibition of chemotaxis by pertussis toxin for most chemokine receptors characterized to date (198).

Following chemokine binding, the chemokine receptor activates the G protein to exchange GDP for GTP, then dissociates into G $\alpha$  and G $\beta\gamma$  subunits {reviewed in (180, 198-200)}. The G $\alpha$  subunit deactivates membrane-bound adenylate cyclase, and subsequently, reduces cyclic adenosine monophosphate (cAMP) levels (201). The recent finding that the G $\alpha$  subunit activates src family kinases provides a potential link between receptor ligation and the tyrosine phosphorylation of numerous downstream effectors like cytoskeletal and focal adhesion proteins (202, 203). While the G $\alpha$  subunit itself is not essential for chemotaxis, its release from the G $\beta\gamma$  subunits is necessary for cell migration (204). The G $\beta\gamma$  subunits activate phospholipase C (PLC)  $\beta 2$  and PLC $\beta 3$ , which in turn, generate inositol-1,4,5-trisphosphate (IP $_3$ ) and the transient release of intracellular calcium stores. In addition to IP $_3$ , PLC induces the formation of diacylglycerol (DAG), which then activates protein kinase C (PKC). Because the PLC pathway had been the most well-characterized biochemical pathway of GPCR, it has served as the primary pathway for assessing responsiveness of chemokine receptors to

different chemokines (198). However, recent evidence suggests that the PLC pathway is not required for chemotaxis. Neutrophils from PLC $\beta$ 2/PLC $\beta$ 3-deficient mice did not exhibit chemokine-induced IP<sub>3</sub> production and calcium efflux, yet migrated normally in response to chemokine stimulation (205). Alternatively, the PLC pathway has proven necessary for chemokine-induced superoxide anion production, protein kinase activation, and adhesion molecule detachment from the matrix (205, 206). This suggests that the chemotactic signals and those leading to cellular activation are mediated through the same receptor, but utilize divergent signaling pathways.

In recent years, type I<sub>B</sub> phosphatidylinositol 3 kinase  $\gamma$  (PI3K) has emerged as a key mediator in chemokine receptor signal transduction (198, 207, 208). The G $\beta\gamma$  subunit directly activates PI3K and results in the formation of phosphatidylinositol 3,4,5-trisphosphate (PtdIns (3,4,5) P<sub>3</sub>) (209, 210). The involvement of PI3K in chemokine signaling was first identified when wortmannin, a PI3K inhibitor, was shown to block RANTES-induced chemotaxis and polarization of T lymphocytes (211). Since that time, studies using wortmannin and PI3K-deficient cells have demonstrated the involvement of PI3K in chemokine-induced adhesion molecule up-regulation, degranulation, superoxide generation, actin reorganization, membrane ruffling, and chemotaxis (205, 207, 212, 213). In a model of septic peritonitis, neutrophils and macrophages of PI3K knockout (-/-) mice exhibited severely impaired recruitment into the peritoneal cavity, as well as the inability to clear bacteria

introduced into the peritoneal cavity (212). However, PI3K-deficiency does not completely abrogate cell migration *in vivo*, suggesting that alternative mechanisms exist to sustain this essential biological process (205, 212, 213).

Recently, several downstream effectors of PI3K have been identified. One downstream effector of PI3K is the serine/threonine protein kinase B (PKB) or Akt (214). PKB is a known mediator of growth factor-induced cell survival and has been shown to regulate the activation of the transcription factor NF- $\kappa$ B (215) (216). While activated PKB has been shown to be localized to the leading edge of a migrating cell, its role in chemotaxis has not been fully elucidated (217). The signaling pathway through which numerous chemokines have been reported to activate the MAPK family of serine/threonine kinases: p38, JNK, and extracellular signal-regulated kinases (ERK1/2), has not been clearly defined (211, 218-221). However, a recent study has demonstrated that the intrinsic protein kinase activity of PI3K can activate the MAPK cascade (222). Further support for the involvement of PI3K in MAPK activation is the profound reduction of chemokine-activated MAPK in PI3K-deficient cells and in normal cells treated with PI3K-inhibitors (213, 219, 221, 223). Another family of downstream effectors believed to be regulated by PI3K is the Rho GTPase family: Rho, Rac, and Cdc42 (207). Their role in regulating the actin cytoskeleton is necessary for cell motility and polarization, key features of migrating leukocytes (224, 225). One study provided evidence for the involvement of PI3K upstream of Cdc42 after demonstrating that wortmannin inhibited cytoskeletal

reorganization stimulated by MCP-1 and MIP-1 $\alpha$ , but not Cdc42 (225).

Additional support for the involvement of PI3K was provided when the expression of inactive variants of PI3K blocked actin reorganization via GPCR (226).

The chemokine receptors, like other GPCR, are rapidly desensitized and internalized after ligand stimulation (227-229). Two distinct types of desensitization have been demonstrated in chemokine receptors: homologous and heterologous desensitization (180, 198). Homologous desensitization occurs when a receptor is rendered refractory to restimulation by prior exposure to the same ligand. In homologous desensitization, the C-terminal serine and threonine residues are phosphorylated by GPCR kinases after ligand binding (188). Phosphorylation allows for the binding of  $\beta$ -arrestin proteins, which sterically inhibit coupling and activation of G $\alpha$  subunits (230, 231). Consequently, the signal transduction process is "switched off" as the G $\alpha$  subunits convert GTP to GDP and reassociate with the G $\beta\gamma$  subunit complex. The  $\beta$ -arrestins also function as adaptor proteins to target ligand-receptor complexes to clathrin-coated vesicles for internalization (231, 232). Chemokine receptors are then resensitized in the acidified endosomal environment and recycled to the plasma membrane (233). In heterologous desensitization, receptor responsiveness to its cognate ligand is diminished by engagement and subsequent activation of an unrelated receptor. Typically, the desensitized receptor is phosphorylated by PKA or PKC, which is triggered by second messengers from an independently activated

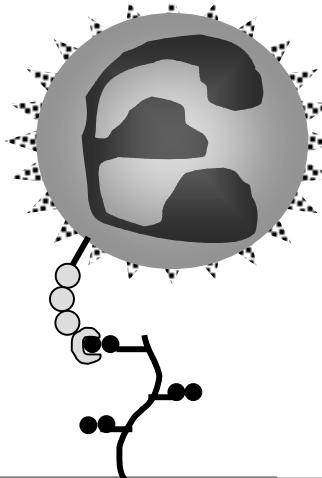
receptor (234). In contrast to homologous desensitization, the heterologously desensitized receptor does not necessarily undergo arrestin-mediated internalization (234). In one demonstration of heterologous desensitization, cells cotransfected with CXCR1 and the C5a receptor were cross-phosphorylated and cross-desensitized after stimulation with either ligand (235). It is generally believed that receptor desensitization is an essential regulatory mechanism for directional migration because it facilitates the rapid detection of alterations in the chemotactic gradient by migrating leukocytes.

### **Chemokines in Leukocyte Trafficking**

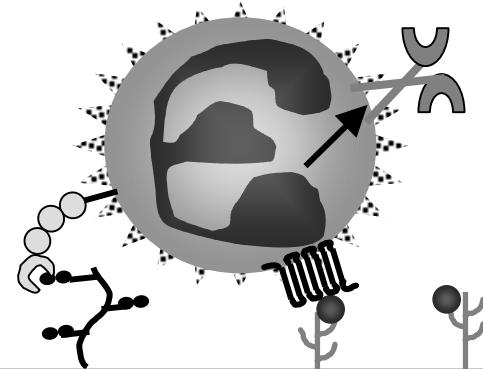
The ability of chemokines to induce the migration of leukocytes from peripheral blood into tissue is regarded as an essential biological role for normal immune surveillance, as well as for the generation of a protective immune response. The multi-step process that leads to leukocyte extravasation involves tethering, activation, adhesion, and transmigration (Figure 5)(73). Each step in this process involves the sequential interaction of numerous molecules and their counterligands to provide combinatorial diversity in signals and outcome. Initially, unactivated leukocytes are "tethered" by transient interactions between selectins and their counterligands on the surface of leukocytes and the vascular endothelium. The selectins are transmembrane glycoproteins with N-terminal, lectin-like domains that mediate cell-to-cell contact through calcium-dependent interactions (236). The elongated and flexible molecular structure of selectins

**Figure 5.** The Role of Chemokines in Leukocyte Trafficking. This diagram illustrates the four-step model for leukocyte recruitment. (1) Initially, leukocytes are tethered by transient interactions between selectins and their counterligands on the surface of leukocytes and the vascular endothelium. This interaction serves to prolong leukocyte contact with the endothelial surface under the shear force of blood flow, thereby increasing the likelihood of exposure to proadhesive molecules like chemokines. Chemokines released from cells near the site of infection bind to proteoglycans on the endothelial cell surface where they are presented to leukocytes. (2) Activation of the leukocyte following chemokine receptor ligation causes integrins on the surface of the leukocyte to undergo conformational changes necessary for high affinity binding to their counterreceptors on the surface of endothelial cells, the cell adhesion molecules (e.g., ICAM, VCAM). (3) The firm adhesion mediated by integrins and cell adhesion molecules results in the arrest of the leukocyte at the surface of the endothelium. (4) Once arrested, the leukocyte marginates between endothelial cell junctions, across the basement membrane, and into the extracellular tissue matrix.

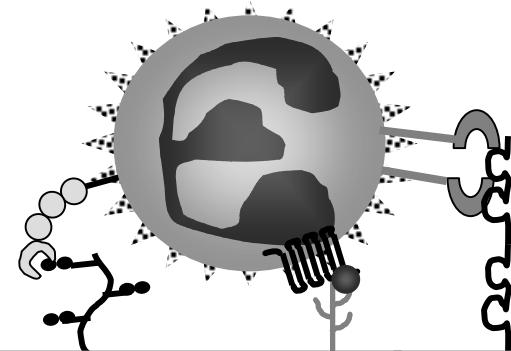
**1. Tethering  
(selectins)**



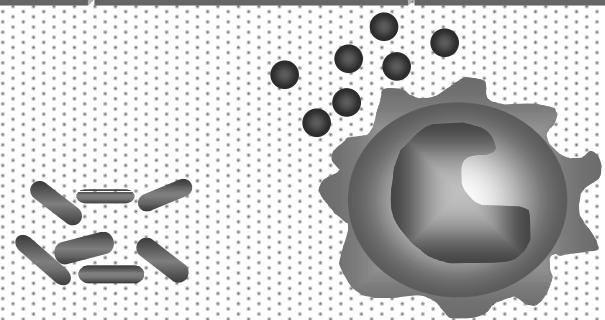
**2. Activation  
(chemokines)**



**3. Adhesion/Arrest  
(integrin/CAM)**



**Site of Infection**



**4. Transendothelial  
Migration**

makes them accessible for interactions with closely opposed cells (237). Within the selectin family, E- and P-selectin are expressed on the surface of activated endothelial cells, while L-selectin is expressed constitutively on the surface of leukocytes (238). Selectins bind via their lectin-like domains to sialylated carbohydrate determinants, such as the tetrasaccharide sialyl Lewis<sup>x</sup> and its isomer sialyl Lewis<sup>a</sup> (239, 240). Their rapid association and dissociation rate constants facilitate "tethering" and "rolling," respectively, along the endothelial surface (237). This interaction is sufficiently strong that it prolongs leukocyte contact with the endothelial surface under the shear force of blood flow, thereby increasing the likelihood of exposure to proadhesive molecules.

Because leukocyte tethering is a reversible process, leukocytes are released back into the circulation unless they encounter additional proadhesive molecules, like chemokines, to activate integrin-mediated adhesion and leukocyte arrest (73). Chemokines were first implicated as the proadhesive signal for integrin activation after pertussis toxin, a GPCR-inhibitor, blocked leukocyte adhesion to endothelium (241). However, it has always been unclear how chemokines can maintain sufficient local concentrations under the shear force of blood flow to activate integrin-mediated adhesion. Instead, it has been proposed that chemokines form a haptotactic, or substrate-bound gradient, due to the ability of the C-terminus of chemokines to bind heparin-bearing proteoglycans on vascular endothelium (103, 104). In this way, chemokines are first immobilized on the luminal surface of the endothelium in close proximity to

the inflammatory site where they can interact with rolling leukocytes. Studies conducted under conditions that simulate blood flow have demonstrated that MIP-3 $\alpha$ , MIP-3 $\beta$ , SLC, and SDF-1 rapidly induce integrin-mediated adhesion of lymphocyte subsets to the surface of endothelial cells (242). Alternatively, MCP-1, RANTES, MIP-1 $\alpha$ , and MIP-1 $\beta$  failed to promote adhesion of T lymphocytes to endothelial cells under flow conditions, yet promoted strong adhesion to the extracellular matrix protein, fibronectin (243). These studies raise the possibility that a distinction exists between chemokines that function at the vascular endothelium versus those in tissues.

Firm adhesion of activated leukocytes to the endothelium is mediated by the integrin family of adhesion molecules. Integrins are heterodimeric transmembrane proteins comprised of noncovalently associated  $\alpha$  and  $\beta$  subunits (237). Leukocyte adhesion to the endothelium is mediated by several integrin family members: (i) the  $\beta_2$  integrins, lymphocyte function associated antigen (LFA-1 or CD11a/CD18), leukocyte adhesion receptor (Mac-1 or CD11b/CD18), and p150/95 (CD11c/CD18); (ii) the  $\beta_1$  integrins, very late antigen (VLA)-1- 6; and (iii) the  $\beta_7$  integrin murine mucosal homing receptor (LPAM-1) (237). Generally, integrins are expressed constitutively on cells, but they can be quickly up-regulated following activation by proinflammatory mediators (*i.e.*, LPS, classical chemoattractants, chemokines, and cytokines). Activation following chemokine receptor ligation causes integrins to undergo conformational changes necessary for high affinity binding to their counterreceptors, a process referred to

a "inside-out" signaling (241). Integrins not only bind to immunoglobulin family members like intercellular adhesion molecule (ICAM)-1, ICAM-2, vascular cell adhesion molecule (VCAM)-1, and mucosal addressin cell adhesion molecule (MAdCAM)-1, but also, they bind to adhesive glycoproteins like fibronectin, fibrinogen, collagen, and von Willebrand factor (237). The firm adhesion mediated by integrins promotes leukocyte margination between endothelial cell junctions, across the basement membrane, and into the extracellular tissue matrix. This process is facilitated by the dramatic cytoskeletal rearrangements of the activated leukocyte. These morphological changes allow for extension and retraction of pseudopods to execute coordinated directional migration along a chemotactic or haptotactic gradient to the site of inflammation.

### **Redundancy versus Specificity**

The prevailing notion that chemokines are functionally redundant has been attributed to several key features of the chemokine superfamily. First of all, there is typically more than one chemokine capable of activating any particular cell population. For example, IL-8, GCP-2, GRO- $\alpha/\beta/\gamma$ , ENA-78, and NAP-2 all recruit neutrophils, while IP-10, SDF-1, MCP-1-3, RANTES, MIP-1 $\alpha/\beta$ , lymphotactin, IL-8, and fractalkine all recruit NK cells (85). Second, a specific cell population can express receptors for multiple chemokines. Monocytes express a diverse repertoire of receptors that bind chemokines from multiple subfamilies (*e.g.*, CXCR2-4, CCR1-5,-8, and CX<sub>3</sub>CR1) (85). Third, a single

chemokine can exert its effects on multiple cell types. The CC chemokine, RANTES, has been shown to act upon monocytes, T cells, NK cells, dendritic cells, mast cells, basophils, and eosinophils (85). Fourth, a specific cell type can produce multiple chemokines in response to the same stimulus. In fact, at least 12 different chemokines (*e.g.*, MIP-1 $\alpha/\beta$ , RANTES, MCP-1-2, MIP-2 $\alpha/\beta$ , IL-8, etc.) were identified as some of the most highly induced transcripts in LPS-stimulated monocytes using a comprehensive and quantitative technique called serial analysis of gene expression (SAGE) (244). Lastly, chemokine-receptor interactions are promiscuous. As previously detailed in the "Chemokine Receptor" section, an individual chemokine receptor can bind multiple chemokines, and an individual chemokine can bind more than one receptor.

While the concept of redundancy was founded largely on data from *in vitro* studies, additional evidence of redundancy has been obtained from *in vivo* studies using neutralizing antibodies, receptor antagonists, and gene knockout mice (76, 189, 245, 246). For example, in a murine model of acute inflammatory lung injury, antibody neutralization of the neutrophil chemoattractants, KC and MIP-2, did not reduce the accumulation of neutrophils into the lungs even though their expression was profoundly elevated in the lung (247). Additionally, mice deficient in CCR5, a receptor for macrophage chemoattractants like MIP-1 $\alpha$ , MIP-1 $\beta$ , and RANTES, were comparable to wild-type mice in their ability to recruit macrophages into the peritoneum following thioglycollate injection (248). Further evidence to support the concept of redundancy was the observation that

humans who lack surface expression of CCR5, as a result of a 32-nucleotide deletion of the CCR5 gene (CCR5 Δ32 allele), did not exhibit a deleterious phenotype (249). Because CCR5 serves as a coreceptor for macrophage (M)-tropic strains of HIV, individuals who are homozygous for the CCR5 Δ32 allele have the selective advantage of resistance to HIV infection (175-177, 249). Collectively, these *in vivo* studies indicate that compensatory mechanisms are capable of overcoming the blockade or deficiency of any one chemokine. It has been proposed that the redundancy of the chemokine system contributes to its "robustness" or ability to maintain certain critical functions of evolutionary importance (245). Alternatively, there may be other highly sophisticated functions of the chemokine system that are less critical for survival, and consequently, not as robust.

Although redundancy exists within the chemokine system, there is a considerable degree of specificity as well. One factor that contributes to selectivity is the differential regulation of chemokine and chemokine receptor expression. Numerous studies have revealed variability in regulation at the level of the inducing stimulus, the developmental stage or activation-state of the target cell, the kinetics of the response, and the microenvironment or location. Chemokines and chemokine receptors have different requirements for their induction. Among the neutrophil chemoattractants, the induction of IL-8 depends on the transcription factors NF-κB and NF-IL-6 or AP-1; whereas GRO $\alpha$  requires the combination of NF-κB and the immediate upstream regulatory (IUR)

element binding factor (250, 251). The inducing stimulus influences the expression of chemokine receptors as well. In human patients with sepsis, neutrophil recruitment in response to CXC chemokines appears to be mediated primarily by CXCR1, since the only other CXC receptor that mediates neutrophil recruitment, CXCR2, is profoundly down-regulated during sepsis (252). The developmental stage and activation-state of the target cell is particularly relevant in the regulation of lymphocytes. For instance, immature double positive (CD4<sup>+</sup> CD8<sup>+</sup>) and double negative (CD4<sup>-</sup> CD8<sup>-</sup>) thymocytes in the thymic cortex respond to SDF-1, mature CD4<sup>+</sup> and CD8<sup>+</sup> thymocytes in the medulla respond to MIP-3 $\beta$ , and activated T cells in the peripheral blood are recruited to the thymus by MDC (84). One example of the variability in temporal and spatial regulation of neutrophil chemoattractants was demonstrated in a murine model of endotoxemia. The mRNA expression of MIP-2 peaked in the lung within 1 hour of LPS administration, while expression of LIX peaked after 4 hours (253). In contrast to the dramatic up-regulation of MIP-2 mRNA expression in the liver, LIX mRNA was only minimally inducible (253).

Because chemokine receptor signaling is dependent upon the combination of ligand, receptor, and target cell type, the resulting variability of response contributes to specificity. For example, ligation of CCR3 on the surface of basophils by RANTES mediates chemotaxis, whereas MCP-1 induces degranulation (87). In another example, human IL-8 binds with high affinity to two highly homologous receptors, CXCR1 and CXCR2. Although ligation of IL-8

to either receptor induces chemotaxis in neutrophils, binding to CXCR1 only results in the activation of phospholipase D and superoxide generation (254). Additionally, the slow internalization and rapid re-expression of CXCR1 after binding IL-8 facilitates continuous signaling, while the rapid internalization and degradation of CXCR2 mediates acute responses (228). Lastly, although RANTES mediates chemotaxis in both eosinophils and basophils via ligation of CCR3, RANTES is only capable of inducing degranulation in eosinophils (87).

While *in vivo* studies using neutralizing antibodies, receptor antagonists, and gene knockout mice have largely substantiated the concept of redundancy in the chemokine system, these studies have also provided evidence to support specificity (76, 189, 245, 246). Among the most compelling evidence to date is the lethality caused by targeted disruption of either SDF-1, or its sole receptor, CXCR4, in mice during late gestation (255-257). Additionally, mice deficient in CXCR2, a receptor that binds neutrophil chemoattractants, exhibit an 80% reduction in their ability to recruit neutrophils into the peritoneum following thioglycollate administration (258). In a murine model of pulmonary *Cryptococcus neoformans* infection, neutralizing antibody to MCP-1 almost completely abrogated the recruitment of monocytes and T cells into the lungs and prevented the clearance of infection (259). Similarly, antibody neutralization of eotaxin dramatically reduced the influx of eosinophils into the lung in a murine model of allergic inflammation (160). Furthermore, pretreatment with truncated GRO $\alpha$  or platelet factor-4 (PF4), receptor antagonists for CXCR2,

substantially reduced neutrophil recruitment into subcutaneous air pouches by CXC chemokines (260). Collectively, these *in vivo* studies demonstrate that the specificity within the chemokine system can be exploited for therapeutic intervention.

### **Thesis Rationale**

The influx of activated leukocytes into host tissues contributes enormously to the pathogenesis associated with LPS. Chemokines have emerged as some of the most potent mediators of leukocyte migration and activation, and consequently, play a pivotal role in the inflammatory response to LPS. However, the literature surrounding LPS-inducibility of chemokines has focused on the role of individual chemokines when, clearly, the process of leukocyte recruitment involves a complex orchestration of multiple cell-specific chemotactic factors in a time-dependent and tissue-specific manner. Additionally, current models suggest that overlapping and perhaps redundant regulatory mechanisms are involved in leukocyte mobilization and tissue infiltration. The purpose of these studies was threefold. First, an extensive panel of LPS-induced CXC and CC chemokines was systematically evaluated in macrophages to enhance our understanding of the cellular and molecular regulatory mechanisms *in vitro* that may contribute to their selective expression and function *in vivo*. Second, I sought to extend my findings *in vitro* to a comprehensive evaluation of chemokine regulation *in vivo*. This series of studies was conducted by analyzing

chemokine gene and protein expression in normal and macrophage-depleted mice after administration of LPS to assess their spatial and temporal regulation in response to LPS, as well as to establish a role for macrophages in their regulation *in vivo*. Third, to determine if therapeutic targeting of a specific cytokine could mitigate endotoxicity, we evaluated mice with a targeted disruption in the gene encoding the chemokine, KC, in a model of LPS-induced lethality.

## MATERIALS AND METHODS

**Reagents.** Protein-free (<0.008%), phenol-water-extracted *Escherichia coli* K235 LPS was prepared according to the method of McIntire et al. (261). Protein-rich (~18%) *E. coli* K235 LPS (but-LPS) was prepared using the butanol-extraction method described by Morrison and Leive (262). Monophosphoryl lipid A (MPL) was kindly provided by RIBI ImmunoChem Research, Inc. (Hamilton, MT). A soluble extract of *Toxoplasma gondii* tachyzoites (STAg) was prepared as described previously and was a gift of Dr. Alan Sher (NIAID, NIH, Bethesda, MD) (263). The synthetic flavone analogue 5,6-dimethylxanthenone-4-acetic acid (MeXAA), a generous gift of Dr. Lai-Ming Ching (University of Auckland, Auckland, New Zealand), was solubilized as detailed previously (264).

Recombinant murine IL-10 and recombinant murine IFN- $\gamma$  were provided by DNAX (Palo Alto, CA) and Genentech, Inc. (South San Francisco, CA), respectively. Tyrphostin AG556 was synthesized as described previously (265) and was kindly provided by Dr. Abraham Novogrodsky (Felsenstein Medical Research Center, Petach Tikva, and Sackler Faculty of Medicine, Tel Aviv University, Israel). A stock solution of AG556 (10 mM) was prepared immediately before use by solubilizing in dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO) and then further diluting in supplemented RPMI 1640 medium containing 2% fetal calf serum (FCS). Liposomes that contain either dichloromethylene bisphosphonate (Cl<sub>2</sub>MBP, Boehringer Mannheim,

Mannheim, Germany) or PBS were prepared as described previously (266) and were kindly provided by Dr. Nico van Rooijen (Vrije Universiteit, Amsterdam, The Netherlands).

**Mice.** C57BL/6J, C3H/OuJ, and C3H/HeJ mice (6-7 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME) and C57BL/6J x 129/Sv F<sub>1</sub> mice (7-12 weeks old) were purchased from Taconic (Frederick, MD). Mice were housed in a laminar flow hood in filter-topped cages and were fed standard laboratory chow and acid water *ad libitum*.

Several strains of mice with targeted mutations ("knockout mice") were also used in these studies. Specifically, IRF-1<sup>-/-</sup> and IRF-2<sup>-/-</sup> mice were generated by targeted mutation as described elsewhere (267). IRF-1<sup>+/+</sup>, IRF-1<sup>-/-</sup>, and IRF-2<sup>+/+</sup>, IRF-2<sup>-/-</sup> breeding pairs, backcrossed to C57BL/6 mice, were provided originally by Dr. Tak Mak (Amgen Institute, Toronto, Canada). The mice used in these studies were the progeny of either homozygotic or heterozygotic matings to obtain background-matched wildtype (+/+ and knockout (-/-) mice, as detailed elsewhere (268). The genotype of every mouse used in this study was confirmed as detailed previously (268). Briefly, mice were bled from the retro-orbital sinus and genomic DNA was purified using a DNA microextraction kit (Stratgene, La Jolla, CA). PCR amplification was performed on genomic DNA (50 -100 ng) with gene-specific and neomycin-specific primers and then amplified products were electrophoresed and resolved on an ethidium bromide-stained 3% agarose gel. STAT1<sup>-/-</sup> and STAT1<sup>+/+</sup> mice were kindly provided by

Dr. Robert Schrieber (Washington University, St. Louis, MO) and have been described in detail previously (269).

Mice with a targeted mutation in the KC gene ( $KC^{-/-}$ ) were provided by Dr. David Bol (Bristol Myers-Squibb, Princeton, NJ). The derivation of these mice has not been reported previously.  $KC^{-/-}$  mice were generated at Bristol Myers-Squibb as follows: murine N51/KC genomic clones were isolated from a genomic library of DNA from 129/Sv mice. A 3.8 kb Xba I-Apa I fragment from the 5' end of the gene was subcloned into pGem 11 zf (Promega, Madison, WI). The fragment was rescued from the plasmid using Xho I/Not I digestion and inserted into the Xho I-Not I site from the pPNT vector, generating pPNT-XN. A 4.8 kb BamHI fragment from the 3' end of the gene was cloned into the Bam HI site of pPNT-XN, generating the final vector pPNTKC/ko. The targeting vector, pPNTKC/ko, contained a neomycin-resistance cassette for positive selection with G418 (Geneticin) and the Herpes simplex virus thymidine kinase gene for negative selection with the uracil derivative of ganciclovir, FIAU (1-(2-deoxy, 2-fluoro- $\beta$ -D-arabinofuranosyl)-5-iodouracil). Orientation of the Bam HI insertion was confirmed by digestion with Xho I/Cla I.

CJ7 embryonic stem (ES) cells, derived from 129/Sv mice, were propagated on primary embryonic fibroblasts as previously described (270). Proliferating feeder cells were inactivated by mitomycin C treatment. Cells were transformed by electroporation with 50  $\mu$ g of pPNTKC/ko that was previously linearized with Not I. Selection of neomycin-, FIAU-resistant ES clones was

performed using 400 µg/ml G418 and 2 µM FIAU (1-(2-deoxy, 2-fluoro- $\beta$ - $\delta$ -arabinofuranosyl)-5-iodouracil). Individual colonies were picked 8 to 12 days after the electroporation and expanded for further use. Targeted clones were identified by Southern blot analysis. Genomic DNA was isolated and digested overnight with KpnI/Eco RV. Samples were resolved on 0.7% agarose gels and transferred onto nylon membranes. The probe consisted of a 0.5 kb fragment isolated from sequences immediately adjacent to the most 3' sequence contained in the targeting vector.

ES cells heterozygous for the targeted locus were injected into C57BL/6J blastocysts. Male chimeric mice were obtained from 2 different clones and bred to C57BL/6J females. Agouti offspring containing a targeted N51/KC locus were intercrossed and yielded the expected 1:2:1 ratio of wild-type, heterozygous, and null mice, respectively, at the KC locus. These data are presented within the Results section. KC<sup>-/-</sup> mice were inbred at Bristol Myers-Squibb, resulting in a mixed C57BL/6 and 129/Sv background. Therefore, C57BL/6J x 129/Sv F<sub>1</sub> mice (7-12 weeks old), purchased from Taconic (Frederick, MD), were used as KC<sup>+/+</sup> controls.

As functional confirmation of the targeted deletion in the KC locus, the expression of KC mRNA was analyzed by Northern blot. Total RNA was isolated from the livers of KC<sup>+/+</sup>, KC<sup>+-/-</sup>, and KC<sup>-/-</sup> mice 4 hours after i.p. injection with 100 µg LPS. Total RNA (10 µg) was fractionated on a denaturing 1% agarose-formaldehyde gel and transferred to nylon membranes by capillary

action. After UV-crosslinking, the membranes were hybridized with a  $\alpha$ -<sup>32</sup>P-dCTP-labeled 0.3 kb ApaI-ClaI fragment from KC cDNA. These data will be presented in greater detail in the Results section.

**Mouse Models.** For kinetic analysis of chemokine gene induction *in vivo*, C57BL/6J mice were injected i.p. with 25  $\mu$ g (~1.25-1.4 mg/kg) LPS. In macrophage depletion studies, C57BL/6J mice were administered 0.2 ml of saline, Cl<sub>2</sub>MBP-liposomes, or PBS-liposomes i.v. on 2 successive days prior to i.p. injection with 25  $\mu$ g of LPS. In each experiment, 4-5 mice were used per timepoint for each treatment. Previous studies have shown that i.v. injection of dichloromethylene bisphosphonate- (Cl<sub>2</sub>MBP) liposomes selectively depletes macrophages from the liver and the splenic red pulp (266, 271, 272). Macrophage depletion was confirmed as detailed previously (68, 266). In studies evaluating KC<sup>-/-</sup> mice administered a lethal dose of LPS, KC<sup>-/-</sup> and age-matched C57BL/6J x 129/SV F1 control mice were injected i.p. with 35 mg/kg (~600 - 1000  $\mu$ g) of LPS. For mortality studies, mice were monitored for morbidity and mortality twice daily for seven days, at which point all surviving mice were euthanized by CO<sub>2</sub> asphyxiation.

**Macrophage Isolation and Culture Conditions.** Peritoneal exudate macrophages were harvested by peritoneal lavage with sterile saline from mice 4 days after i.p. injection with 3 ml of sterile 3% thioglycollate broth. The cells were washed and resuspended in RPMI 1640 supplemented with 2% FCS, 7.5%

sodium bicarbonate, 10 mM HEPES, 2 mM glutamine, and 100 U/ml penicillin-100 µg/ml streptomycin. For culture supernatants and isolation of RNA, cells were plated in 6-well plates at a final concentration of  $4 \times 10^6$  cells/well in 2 ml of medium. Following overnight incubation at 37° C, nonadherent cells were removed by washing, and adherent monolayers were treated in a final volume of 2 ml as indicated. Macrophage culture supernatants were harvested at indicated time points and stored at -70° C until analyzed for protein production by ELISA (see below). Remaining adherent monolayers were used for RNA isolation as detailed below.

**Isolation of Total Cellular RNA.** For peritoneal macrophage cultures, supernatants were removed after treatment and cells were lysed with 1 ml/well (6-well plate) of RNA Stat-60 (Tel-Test, Inc., Friendswood, TX). For *in vivo* studies, samples of organ tissue (~100 mg) were harvested at indicated time points following treatment, snap-frozen in an ethanol-dry ice bath, and stored at -70° C. Frozen organ samples were thawed on ice and immediately homogenized in RNA Stat-60. Total RNA was isolated as specified by the manufacturer and the concentration of RNA was determined by spectrophotometric analysis.

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR).** Coupled RT-PCR was used to determine the relative quantities of mRNA for each gene of interest (273). Briefly, 1 µg of total RNA was denatured at 70° C for 5 min and

then cooled to 4° C for 5 min. For complementary DNA (cDNA) synthesis, a reaction mixture containing 200 U of M-MLV reverse transcriptase (Gibco BRL, Gaithersburg, MD), 20 U/ml of random hexamers, 0.25 mM of deoxynucleotide triphosphates (dNTPs) (Pharmacia, Piscataway, NJ), 20 U of RNasin (Promega), and RT buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl<sub>2</sub>, 8 mM dithiothreitol) (Gibco BRL), was added to the denatured RNA and incubated at 37°C for 60 min. Next, the reaction was heated to 90° C for 5 min, cooled to 4° C, and then diluted 1:8 with double distilled H<sub>2</sub>O. An aliquot of cDNA representing an equivalent of 50 ng of input RNA was used for PCR amplification in a reaction mixture containing 1 U *Taq* polymerase (Promega), 0.2 mM specific sense and antisense primers (Table I), 0.25 mM dNTPs, 1.5 mM MgCl<sub>2</sub>, and PCR buffer (50mM KCl, 10 mM Tris-HCl, pH 9.0, and 0.1% Triton X-100). PCR cycling was performed in an automated Peltier Thermal Cycler (PTC)-100 (MJ Research, Inc., Watertown, MA), with each cycle consisting of 1 min of denaturation at 95° C, 1 min of primer annealing at a gene specific annealing temperature (Table I), and 2 min of primer extension at 72° C, following an initial 3 min denaturation at 95° C. The primer and probe sequences for the following genes have been published previously: glyceraldehyde-3-phosphate (GAPDH) (274); IFN- $\gamma$  (275); IL-1 $\beta$  (276); IL-10 (277); IL-12p35 and IL-12p40 (278); IL-15 (279); and IP-10, JE/MCP-1, KC, MIP-1 $\alpha$ , MIP-1 $\beta$ , MIP-2, and RANTES (159); hypoxanthine-guanine phosphoribosyl transferase (HPRT) and iNOS (273). The primer and probe sequences for CXCR2, IL-18, and TNF- $\alpha$  were selected using

Primer3 ([http://www-genome.wi.mit.edu/cgi-bin/primer/primer3\\_www.cgi](http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi)).

The optimal cycle number for each gene was determined empirically and was defined as the number of cycles that resulted in detectable PCR-amplified product under non-saturating conditions (Table I).

**Detection and Quantitation of PCR Products.** Following electrophoresis of a 20 µl sample of each PCR product on a 1% agarose-TAE gel, the gel was denatured (1.5 M NaCl; 0.5 N NaOH) for 30 min, and then neutralized (0.5 M Tris-HCl, pH 7.0; 1.5 M NaCl) for 30 min. The PCR-amplified products were transferred by capillary action to Hybond N+ membrane (Amersham, Arlington Heights, IL) in 10X SSC by standard Southern blotting procedures. DNA was UV-crosslinked in a Stratalinker 1800 (Stratagene, La Jolla, CA), baked at 80° C for 18 h, and hybridized with a fluoresceinated-internal oligonucleotide probe. Probe labeling and detection was achieved with an enhanced chemiluminescence system (ECL, Amersham). Chemiluminescent signals were detected by Kodak X-Omat AR 5 film, scanned (Datacopy GS plus, Xerox Imaging Systems, Sunnyvale, CA), and densitometric analysis was performed using NIH Image 1.57 software. To quantify the magnitude of change in gene expression, a standard curve was generated from the serial dilution of PCR-amplified cDNA known to be positive for the transcript of interest. The resulting signal from each dilution was plotted and fit to a standard curve by linear regression. The relative expression levels in test samples were calculated with the line equation from the standard curve. Normalization for the relative quantity of mRNA was accomplished by

**Table I.** PCR Oligonucleotide Primer and Probe Sequences, Annealing Temperatures (AT), and Cycle Numbers

Gene AT	Sequence (5'→3')	Cycle Number			Gene AT	Sequence (5'→3')			Cycle Number			
		M	V	G		M	V	G	M	V	G	
CXCR2 60°C	S A P GTCTACCTGCTAACCTGGCC GGTTGTAGGGCAGCC GCCATTGTACATGCCACAAG	na	37	38	iNOS 65°C	S A P CCCTTCCGAAGTTCTGGCAGCAGC GGCTGTCAGAGCCTCGTGGCTTG CAAGGTCTACGTTCAGGACATC	na	35	29			
GAPDH 54°C	S A P CCATGGAGAAGGCTGGGG CAAAGTTGTATGGATGACC CTGCACCACCAACTGCTTAG	25	na	na	IP-10 55°C	S A P GTGTTGACATCATGCCACG GCTTACAGTACAGAGCTAGG TTAACTGGAGAGAACGCCACG	28	24	28			
HPRT 54°C	S A P GATTCAACITGGCTCATCTTAGGC GTTGGATAACAGGCCAGACTTTGTG GTTGTTGGATATGCCCTTGAC	24	24	24	JE 60°C	S A P CTCACCTGCTGCTACTCATTC GCTTGAGGTGGTTGTGGAAAAA CTCATTACCCAGCAAGATGA	28	29	31			
IFN-γ 55 °C	S A P TGCATCTGGCTTGCAGCTC CTTGCTGTTGCTGAAGAAGG GGAGGAACTGGAAAAGGA	na	31	27	KC 65°C	S A P AACGG AGAAAGAAGACAGACTGCT GACGAGACCAGGAGAACAGGG GTGAACGCTGGCTCTGACA	28	36	38			
IL-1β 54 °C	S A P GGGATGATGATGATAACCTG TTGTCGTTGCTTGGTCTCCT CAGCTGCACTACAGGCTCCG	na	30	24	MCP-5 55°C	S A P AGCITTCATTGCAAGTCTTG CTCCTTATCCAGTATGGTCC CAGTCCTCAGGTATTGGCTGG	31	33	28			
IL-10 54°C	S A P CGGGAAGACAATAACTG CATTTCCGATAAGGCTTGG GGACTGCCTTCAGCCAGGTGAAGACTTT	na	29	29	MIP-1α 55°C	S A P CCCAGCCAGGTGTCATTTCC GCATTCAAGTCCAGGTCACTG TGCCTGACTCCAAAGAGAC	24	27	27			
IL-12p35 54°C	S A P GGCTACTAGAGAGACTTCTCC GTGAAGCAGGATGCAGAGCTTC GCAGATCATTCTAGACAAGGGC	na	38	35	MIP-1β 65°C	S A P CCCTCTCTCCTCTTGCTCGT TTCAACTCCAAGTCACTCATGTACTCA AAAGAGGCAGACAGATCTGTGCTAAC	28	34	30			
IL-12p40 60°C	S A P ATCGTTTGCTGGTGTCTCC AGTCCCTTGGTCCAGTGTG AGCAGTAGCAGTCCCCCTGA	na	36	34	MIP-2 65°C	S A P AGTTTGCCTGACCCCTGAAGCC TGGGTGGGATGTAGCTAGTTCC CCTGATGTGCCTCGCTGTCTG	27	40	29			
IL-15 55°C	S A P CCATCTCGTACTTGTG CTGTTGCAAGGTAGAGCAGC TTGGGCTGTGTCAGTGTAG	na	40	36	RANTES 65°C	S A P GCGGGTACCATGAAGATCTCTG CACTTCTCTCTGGGTTGGCAC GCAGTCG TGTTGTCACTCGAA	26	26	28			
IL-18 60°C	S A P ACTGTACAACCGCAGTAATACGG AGTGAACATTACAGATTATCCC AGTGCCAGTGAACCCCCAGAC	na	40	38	TNF-α 60°C	S A P GGG ATG AGA AGT TCC CAA ATG CTCCAGCTGGAAGACTCCTCCCAG TCACACTCAGATCATCTTCTC	30	31	32			

comparison to either GAPDH or HPRT "housekeeping genes" as already described (273, 274). Increases in mRNA were expressed as mean-fold induction relative to untreated controls, which were arbitrarily assigned a value of 1. This precludes comparison of basal gene expression between organs, as well as among the different genes analyzed.

**Blood Collection and Preparation for Analysis.** Mice were euthanized by CO<sub>2</sub> asphyxiation and bled by cardiac puncture at the indicated times after administration of LPS. Blood collected for serum was allowed to stand 2-4 hr at room temperature to facilitate clot formation and then placed at 4° C overnight to allow for the clot to retract. After pelleting the clot by centrifugation at 2,700 × g at 4°C, the serum was collected. Serum for aspartate aminotransferase (AST) and alanine aminotransferase (ALT) analysis was stored at 4° C for no longer than 48 h prior to analysis on a Kodak Ektachem 250 Analyzer (Johnson & Johnson Clinical Diagnostics). Serum for cytokine, chemokine, or nitric oxide (NO) analysis was stored at -70° C until the assay was performed. Blood for white blood cell differential analysis was collected in EDTA-coated microtubes and analyzed the same day on the Cell-Dyn 3500R (Abbott Laboratories, Chicago, IL) using REVH, version 4.2, software.

**Chemokine and Cytokine ELISAs.** Protein concentrations in cell culture supernatants and serum were measured by ELISA. Murine JE/MCP-1, IFN- $\gamma$ , and IL-12p70 OPT-EIA kits (Pharmingen, San Diego, CA); murine MIP-1 $\alpha$  and

IL-18 Quantikine kits (R&D Systems, Minneapolis, MN); and the murine TNF- $\alpha$  Duo-Set kit (Genzyme, Cambridge, MA), were used to detect the respective proteins according to the manufacturer's instructions. Murine KC and MIP-2 were detected using matched antibody pairs (R&D Systems). Briefly, Nunc-Immuno microtiter plates (Nunc A/S, Roskilde, Denmark) were coated with "capture" antibody (1  $\mu$ g/ml of rat anti-mouse KC IgG<sub>2a</sub> or 1  $\mu$ g/ml of goat anti-mouse MIP-2 IgG, R&D Systems) diluted in PBS, pH 7.5. After overnight incubation at 4° C, the plates were washed four times with PBS containing 0.05% Tween-20 and then blocked with PBS containing 1% BSA (PBS-BSA) for 2 h at 25° C. The plates were washed four times and then 100  $\mu$ l of samples and standard (recombinant murine KC or MIP-2, R&D Systems), diluted in PBS-BSA, were added to the plate. Following a 1 h incubation at 25° C, the plates were washed four times and 100  $\mu$ l of "detect" antibody (0.5  $\mu$ g/ml biotinylated goat anti-mouse KC or MIP-2 IgG), diluted in PBS-BSA, was added for 1 h at 25° C. After washing the plates four times, horseradish peroxidase-strepavidin conjugate (16.7 ng/ml in PBS-BSA; Jackson Immunoresearch Laboratories, West Grove, PA) was added. Following a 20 min incubation at 25° C, the plates were washed four times and 100  $\mu$ l of tetramethylbenzidine-hydrogen peroxide substrate, prepared according to manufacturer's instructions (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD), was added for 30 min at 25° C. The reaction was terminated following the addition of 100  $\mu$ l of 1 M H<sub>3</sub>PO<sub>4</sub> and the absorbance was read at 450 nm on an automated microtiter plate reader.

Regression analysis of the standard curve was used to calculate protein concentrations. The lower limit of detection for IFN- $\gamma$ , IL-12p70, IL-18, JE/MCP-1, KC, MIP-1 $\alpha$ , MIP-2, and TNF- $\alpha$  was 15.6, 3.9, 15.6, 15.6, 15.6, 4.7, 15.6, and 15 pg/ml, respectively, as specified by the manufacturer.

**Detection of Nitric Oxide.** Secretion of NO into culture supernatants by macrophages was determined by measuring nitrite as described previously (280). Supernatants were mixed with an equal volume of Greiss reagent (1:1, 0.1% N-(naphyl)ethylenediamine dihydrochloride in H<sub>2</sub>O: 1% sulfanilamide in 5% H<sub>3</sub>PO<sub>4</sub>) and absorbance was measured at 550 nm. Nitrite concentrations were calculated by regression analysis of a NaNO<sub>2</sub> standard curve (1 – 20  $\mu$ M). For detection of NO in serum, nitrate was converted to nitrite with *Aspergillus niger* nitrate reductase (Boehringer Mannheim, Indianapolis, IN) as detailed elsewhere (281). Briefly, 50  $\mu$ l of serum was added to an equal volume of reaction mixture containing 0.05 U/ml nitrate reductase, 4  $\mu$ M flavin adenine dinucleotide, 40  $\mu$ M of the reduced form of nicotinamide adenine dinucleotide phosphate, and 40 mM Tris, pH 7.9. Following a 15 min incubation at 37° C, the reduced samples were mixed with an equal volume of Greiss reagent and absorbance was measured at 550 nm. The total nitrate/nitrite concentration was determined by regression analysis of a reduced NaNO<sub>3</sub> standard curve (1 – 20  $\mu$ M).

#### **Flow Cytometric Analysis of Bone Marrow, Thymocytes, and Splenocytes.**

Relative numbers of granulocytes, monocytes, B and T cells were determined

using FACS analysis for the detection of specific cell surface markers. Bone marrow cells were obtained after flushing the femurs of KC<sup>+/+</sup> and KC<sup>-/-</sup> mice with HBSS. Thymocytes and splenocytes were obtained by disrupting the tissue in HBSS with a metal grid and then filtering through a Nitex membrane. Approximately 5 x 10<sup>5</sup> cells were directly stained with R-phycoerythrin (PE)-labeled or FITC-conjugated monoclonal Abs to Gr-1 (granulocytes), Mac-1 (monocytes, granulocytes, and NK cells), B220 (B cells), and CD3 (T cells) (Pharmingen) in 100 µl of HBSS containing 10% FBS for 30 min on ice. PE- and FITC-conjugated isotype-matched Ig (Pharmingen) were used for controls. Flow cytometry was carried out using a Coulter Epics Profile II flow cytometer and cell sorter. An average of 10,000 cells were recorded in each determination.

**Myeloperoxidase Assay.** Samples of organ tissue (~50 - 100mg) were harvested at indicated time points following treatment, rinsed in PBS, blotted dry, and weighed. Samples were homogenized in 50 mM sodium phosphate buffer (pH 6) and the homogenate was centrifuged (20,000 x g) for 15 min at 4° C. The pellet was resuspended in a 10X volume of 0.5% hexadecyltrimethyl ammonium bromide in 50 mM sodium phosphate buffer (pH 6) and homogenized on ice. The homogenate was freeze-thawed (20 min at -70° C), centrifuged (20,000 x g) for 15 min at 4° C, and the supernatant was stored at -70° C. Myeloperoxidase (MPO) activity in supernatants was measured using a standard spectrophotometric procedure (282). The supernatant was diluted 1:30 in a reaction mixture containing 0.167 mg/ml o-dianisidine and 0.0005% hydrogen

peroxide in a 100 mM potassium phosphate buffer (pH 6). The change in optical density at 460 nm over 2.5 min was measured in a Beckman DU-64 Spectrophotometer using the Soft PAC Kinetics Module. Data were derived by linear regression and are expressed as optical density at 460 nm per minute per gram of tissue.

**Isolation of Hepatic Leukocytes.** Leukocytes were obtained from the livers of control and LPS-injected (35 mg/kg) C57BL/6J x 129/SV F1 and KC <sup>-/-</sup> mice as described previously (283). Briefly, livers were perfused with Hank's Buffered Saline Solution (HBSS), excised, and disrupted in ice-cold HBSS for 1 min in a stomacher (Tekmer, Cincinnati, OH) to generate single cell suspensions. Cells were filtered through 100  $\mu$ m nylon mesh, washed, and resuspended in HBSS containing metrizamide (Nycomed, Oslo, Norway) at a final concentration of 17.5%. The liver cell suspensions were overlaid with HBSS and centrifuged at 1400  $\times$  g for 20 min at 4°C. Nonparenchymal cells were harvested from the interface, washed, and resuspended in HBSS.

**Flow Cytometric Analysis of Hepatic Leukocytes.** Leukocytes were added to 96-well U-bottomed polypropylene plates (Fisher) at a concentration of  $1 \times 10^5$  cells per well. After washing with PBS containing 1% BSA, cells were directly stained with optimally-titrated R-phycoerythrin (PE)-labeled anti-DX5 and FITC-labeled anti-CD3 Abs (Pharmingen) in 100  $\mu$ l of PBS containing 1% BSA for 30 min at 4° C. R-PE- and FITC-conjugated isotype-matched Ig (Pharmingen) were

used for controls. After washing three times in PBS containing 1% BSA, two-color analysis was performed on a FACSort flow cytometer (Becton Dickinson, Mountain View, CA) using Lysys II Ver 2.0 Software. A total of 10,000 cells were analyzed for each experimental group. The percentage of cells expressing each phenotypic marker was determined on cells pooled from 3 livers per group. The absolute number of cells expressing each phenotypic marker was calculated by multiplying the percentage by the mean of the total number of cells isolated from the pooled livers.

**NK Cell-Enriched Cultures for IFN- $\gamma$  Production.** Hepatic leukocytes were enriched for NK cells and T lymphocytes (> 95%) by nylon wool depletion of adherent macrophages and B lymphocytes. NK cells were expanded from the nonadherent cells by culturing for 6 days in complete RPMI-1640 medium (RPMI-1640 containing 10% FBS, L-glutamine, penicillin/ streptomycin, sodium pyruvate, nonessential amino acids, HEPES, and 2-mercaptoethanol) and 1000 Cetus units (CU/ml) of IL-2 (284). Following IL-2 propagation, cells were washed twice and plated at a concentration of  $1 \times 10^6$  cells/well in 24-well plates. Cells were treated in a final volume of 1 ml of complete RPMI-1640 medium alone or in the presence of 10 ng/ml of phorbol myristate acetate and 1 $\mu$ g/ml of ionomycin (PI), 30  $\mu$ g/ml LPS, 3  $\mu$ g/ml LPS, or 1  $\mu$ g/ml rKC (R&D Systems). Cell-free culture supernatants were harvested after 24 h and stored at -70° C until assayed for IFN- $\gamma$  by ELISA (R&D Systems). The distribution of cell subsets,

DX5<sup>+</sup>/CD3<sup>-</sup> (NK), DX5<sup>+</sup>/CD3<sup>+</sup> (NK/T), and DX5<sup>-</sup>/CD3<sup>+</sup> (T), was verified by flow cytometric analysis as described above.

**Statistics.** Comparisons between two groups were analyzed using paired or unpaired Student's *t* test and ANOVA. The accepted level of significance was P < 0.05. The statistical methods used for the analysis of the data presented herein were reviewed and approved by Dr. David Cruese, Department of Preventive Medicine, Biometrics Section, Uniformed Services University of Health Sciences at the beginning of these studies.

## RESULTS

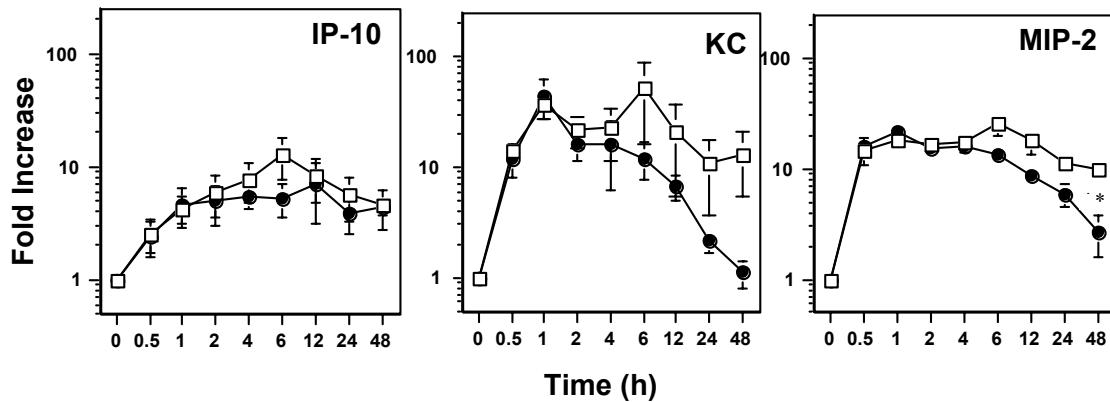
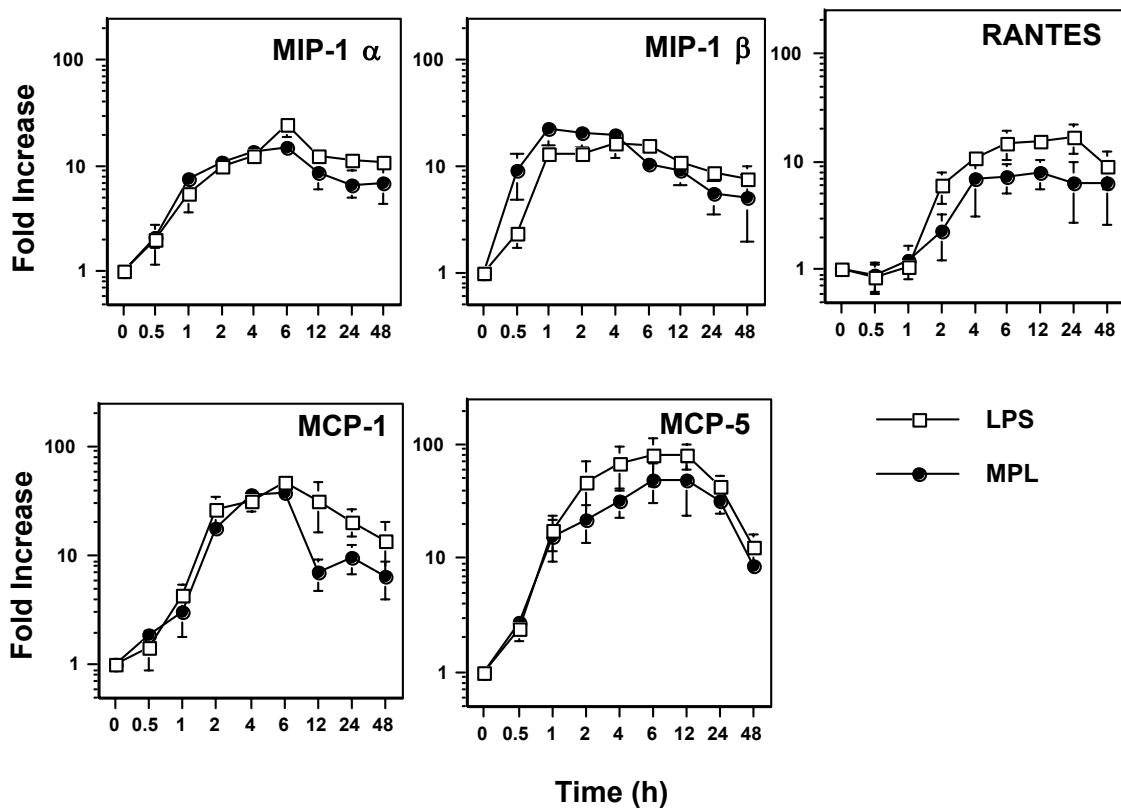
### A. *In Vitro* Analysis of LPS-Induced Chemokine Expression in Murine Peritoneal Macrophages

Recent studies of chemokine expression in response to microbial challenge or microbial products *in vivo* have revealed a much more restricted pattern of expression than would be predicted from analysis of chemokine expression *in vitro*. Additionally, while several different chemokines have been detected during systemic sepsis, numerous reports have demonstrated non-redundant roles for individual chemokines (71, 154, 173, 285). Therefore, the primary goal of these *in vitro* studies was to evaluate a more extensive panel of CXC and CC chemokine genes elicited in response to LPS to enhance our understanding of the cellular and molecular mechanisms that may contribute to their selective expression and function *in vivo*. To this end, we analyzed the regulation of LPS-induced expression of the CXC chemokines, IP-10, MIP-2 and KC, as well as the CC chemokines, JE/MCP-1, MCP-5, MIP-1 $\alpha$ , MIP-1 $\beta$ , and RANTES. While LPS-inducibility of these chemokines had been reported previously (105), and some chemokines were even originally isolated as LPS-induced proteins (*i.e.*, MIP-1 $\alpha$ , MIP-1 $\beta$ , and MIP-2) (70, 118), a comprehensive and systematic approach to evaluating their induction by LPS *in vitro* has not been undertaken to date. Furthermore, because the interaction of LPS with macrophages is pivotal in eliciting the proinflammatory cascade associated with sepsis (63, 286), our *in vitro*

studies sought to characterize the regulation of LPS-induced chemokine gene expression in peritoneal macrophages.

**Kinetic analysis of CXC and CC chemokine mRNA expression induced by LPS and MPL.** We first examined the time course of steady-state mRNA expression for a panel of chemokines in response to LPS and a nontoxic derivative of the lipid A moiety of LPS, MPL (Figure 1B). Like LPS, MPL functions as both an immunostimulant and an inducer of endotoxin tolerance (287-290), yet MPL is significantly less toxic than LPS (291). The attenuated toxicity of MPL has been attributed, in part, to its reduced capacity to induce proinflammatory cytokines like TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-12, and IFN- $\gamma$  (289, 290, 292), as well as its enhanced capacity to induce anti-inflammatory cytokines like IL-10 (292), relative to LPS. To determine whether chemokine mRNA expression was differentially modulated by LPS or MPL over time, C3H/OuJ peritoneal macrophage cultures were incubated with 200 ng/ml LPS or MPL from 0.5 to 48 h. This dose was chosen because it had been shown to be optimal for stimulation in previous studies. As shown in Figures 6A and 6B, steady-state mRNA expression of all CXC and CC chemokines, except for RANTES, was induced 2-20-fold as early as 0.5 h by both LPS and MPL. The induction of RANTES mRNA over basal levels was not observed until 2 h post-LPS or MPL treatment. Steady-state mRNA for KC, MIP-2, and MIP-1 $\beta$  reached peak levels (~15- to 60-fold) as early as 1 to 2 h after LPS or MPL treatment, while IP-10, MIP-1 $\alpha$ , RANTES, JE/MCP-1, and MCP-5 mRNA required 6 h before peak levels (~10- to 90-fold)

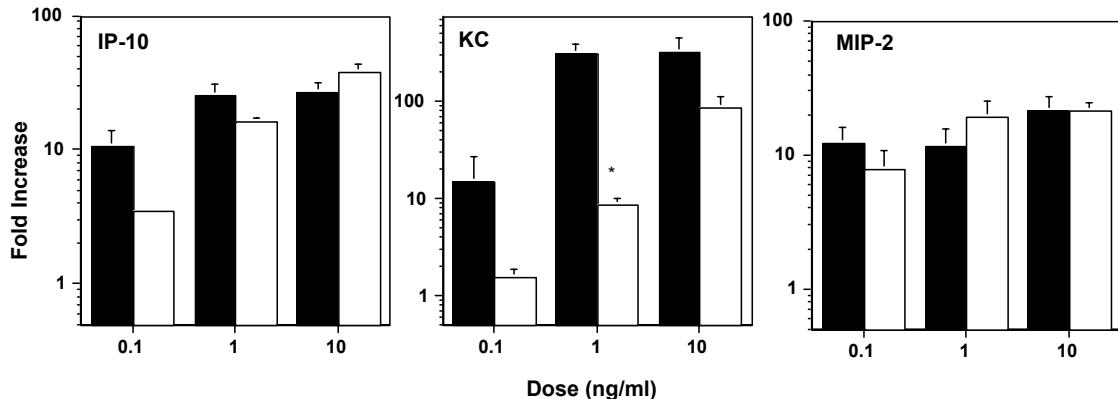
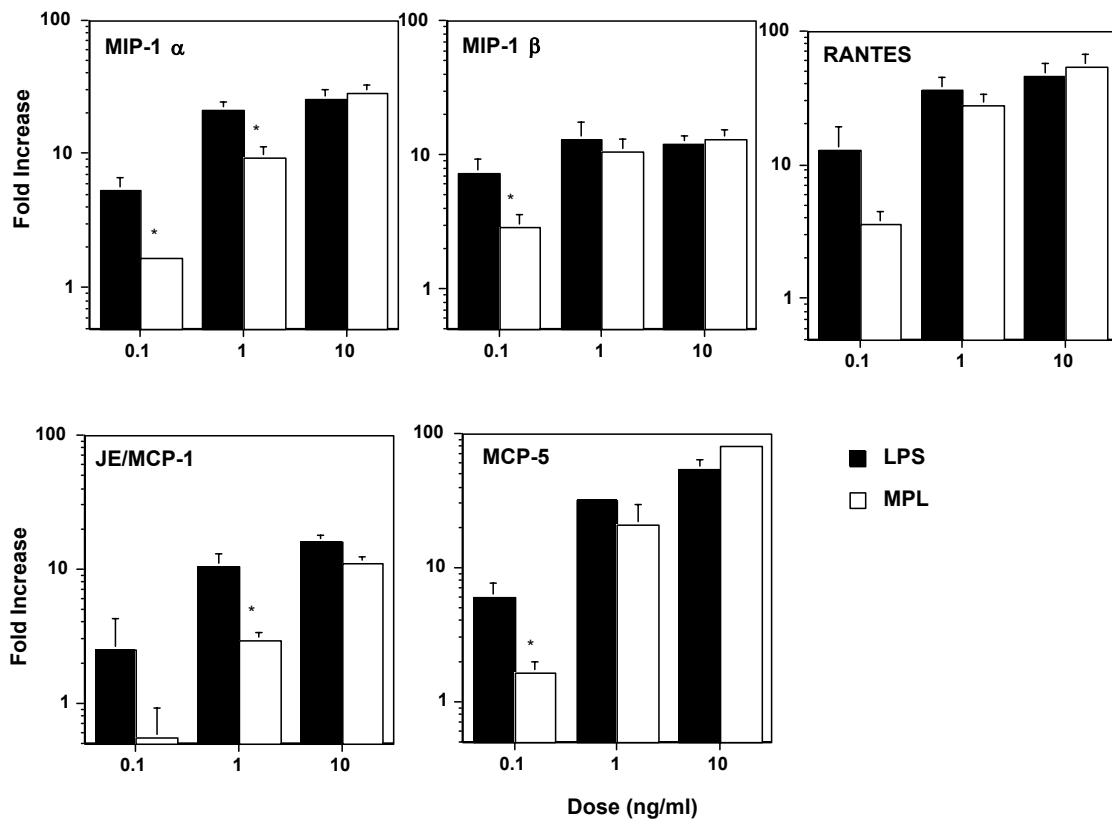
**Figure 6.** Kinetics of LPS- and MPL-induced expression of CXC (A) and CC (B) chemokine mRNA. C3H/OuJ macrophage cultures were treated with 200 ng/ml LPS or MPL for indicated times. Coupled RT-PCR was used to determine the relative quantities of mRNA for each gene of interest after normalization to either GAPDH or HPRT as described in *Materials and Methods*. The magnitude of change in mRNA levels are expressed as the mean fold induction relative to untreated controls (assigned a value of 1)  $\pm$  standard error of the mean (SEM) from 3 or 4 independent experiments. An asterisk indicates MPL-induced mRNA levels are significantly lower ( $p < 0.05$ ) than LPS-induced mRNA levels. When not visible, error bars are smaller than the symbol.

**A. CXC****B. CC**

were achieved. In general, CXC and CC chemokine mRNA expression remained sustained for 48 h with only a gradual decline in steady-state mRNA from peak levels. Interestingly, the kinetics of chemokine mRNA expression in response to MPL closely paralleled the kinetics in response to LPS. The only notable difference observed was a somewhat accelerated decline of KC and MIP-2 mRNA levels at 24 and 48 h after treatment with MPL.

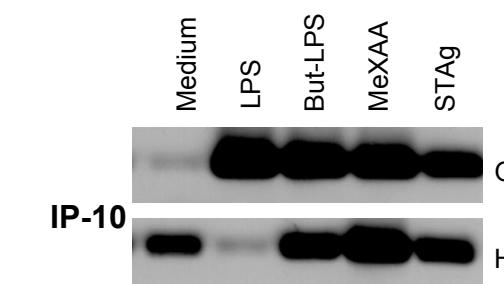
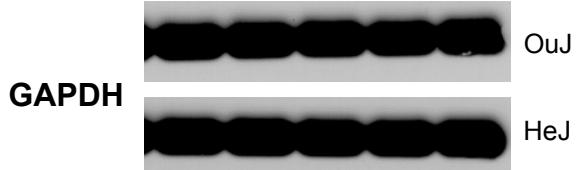
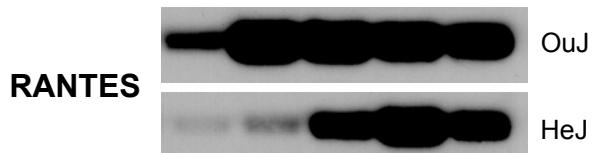
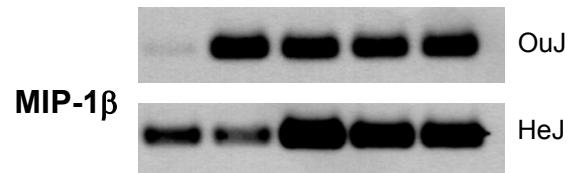
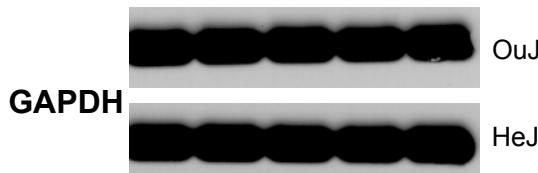
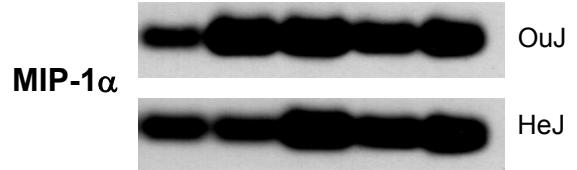
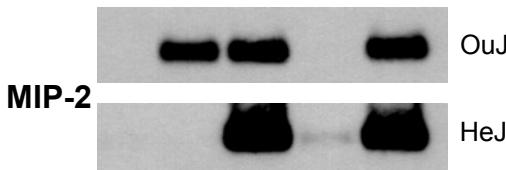
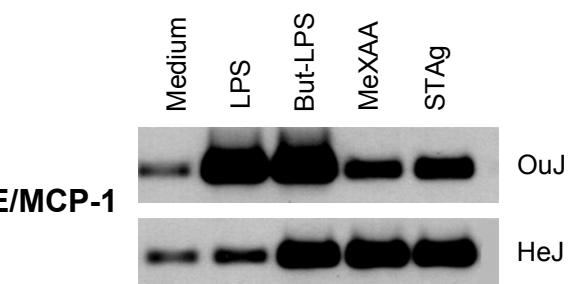
**Dose response analysis of CXC and CC chemokine mRNA expression induced by LPS and MPL.** To evaluate the sensitivity of chemokine mRNA induction by LPS or MPL, C3H/OuJ peritoneal macrophage cultures were incubated with doses of LPS or MPL ranging from 0.1 to 1000 ng/ml. Total cellular RNA was harvested at a time when peak levels of steady-state mRNA for the gene of interest was observed (*i.e.*, 2 h for KC, MIP-1 $\beta$ , and MIP-2; and 6 h for IP-10, MIP-1 $\alpha$ , JE/MCP-1, MCP-5, and RANTES). As shown in Figure 7A and 7B, steady-state mRNA expression for all of the CXC and CC chemokines was induced over basal levels (assigned a value of 1) by as little as 0.1 ng/ml LPS and maximal mRNA expression was achieved with 1-10 ng/ml LPS. No additional increases in chemokine mRNA expression were observed at doses of 100 - 1000 ng/ml of LPS or MPL (data not shown). While LPS was a more potent inducer than MPL at low concentrations (0.1 or 1 ng/ml), the inductive ability of MPL for this panel of genes was remarkably comparable to LPS at higher concentrations (10 to 1000 ng/ml).

**Figure 7.** Dose response analysis of LPS- and MPL-induced expression of CXC (A) and CC (B) chemokine mRNA. C3H/OuJ macrophage cultures were treated with medium or 0.1 to 10 ng/ml LPS or MPL. Total cellular RNA was harvested after treatment when peak mRNA levels were observed for the gene of interest (i.e., 2 h for KC, MIP-1 $\beta$ , and MIP-2; and 6 h for IP-10, MCP-1, MCP-5, MIP-1 $\alpha$ , and RANTES). Data are expressed as the mean fold induction  $\pm$  SEM from 3 or 4 independent experiments. Basal levels were assigned a value of 1. An asterisk indicates MPL-induced mRNA levels are significantly lower ( $p < 0.05$ ) than LPS-induced mRNA levels. When not visible, error bars are smaller than the symbol.

**A. CXC****B. CC**

**Induction of CXC and CC chemokine mRNA by macrophage activating agents other than LPS does not require a normal *Lps* gene product.** Previous studies have taken advantage of the finding that the LPS-hyporesponsiveness exhibited by C3H/HeJ mice is highly specific for protein-free preparations of LPS (293). Alternatively, LPS preparations rich in endotoxin-associated proteins (*i.e.*, but-LPS; (294)), or the purified proteins themselves (294, 295), are potent stimuli in both *Lps<sup>n</sup>* and *Lps<sup>d</sup>* macrophages and induce expression of a subset of LPS-inducible cytokine genes and tyrosine phosphorylation of MAP kinases (296). The molecular basis for the LPS-unresponsiveness exhibited by the C3H/HeJ strain is now understood. A point mutation within the intracytoplasmic domain of the gene that encodes the transmembrane signaling receptor, TLR4, was recently identified in this mouse strain (37, 38). This mutation precludes signaling by protein-free enterobacterial LPS preparations (297). In contrast, protein-rich LPS preparations elicit signaling via both TLR2 and TLR4 (297), thus accounting for the observed, limited signal transduction in C3H/HeJ macrophages that was originally reported by Hogan (294) and Manthey (298, 299). Like endotoxin-associated proteins, a soluble extract of *T. gondii* tachyzoites has been reported to elicit a similar response pattern to that induced by endotoxin-associated proteins, whereas the antitumor agent, MeXAA, elicits induction of a distinct subset of genes in the apparent absence of MAP kinase activation (264) in both *Lps<sup>n</sup>* and *Lps<sup>d</sup>* macrophages. Therefore, to investigate further the inducibility of chemokine genes, C3H/OuJ and C3H/HeJ

**Figure 8.** Differential induction of CXC (A) and CC (B) chemokines in response to macrophage-activating agents in macrophages from LPS-normoresponsive (*Lps<sup>n</sup>*) C3H/OuJ (OuJ) and LPS-hyporesponsive (*Lps<sup>d</sup>*) C3H/HeJ (HeJ) mice. Macrophage cultures were treated with medium, LPS (10 ng/ml), but-LPS (10 µg/ml), MeXAA (100 µg/ml), or STAg (5 µg/ml). Total RNA was harvested after 6 h. A representative Southern blot of 3 independent experiments is shown.

**A. CXC****B. CC**

macrophage cultures were incubated for 6 h with LPS, protein-rich but-LPS, MeXAA, or STAg. The representative Southern blots shown in Figure 8A and 8B illustrate the inducibility profiles observed for the CXC and CC chemokines. As expected, the induction of chemokine mRNA expression by protein-free LPS was limited to C3H/OuJ macrophages, while the protein-rich, but-LPS strongly induced chemokine mRNA expression in C3H/HeJ as well as C3H/OuJ macrophages, except in the case of IP-10 and MCP-5 where induction of gene expression was substantially greater in C3H/OuJ macrophages. Of the CXC chemokines, KC and MIP-2 mRNA exhibited identical patterns of induction. Specifically, both were strongly induced (>100-fold) by STAg, but poorly modulated by MeXAA in C3H/OuJ and C3H/HeJ macrophages (Figure 8A). Unlike the other CXC chemokines, IP-10 mRNA expression was induced 30-40-fold by MeXAA, in addition to being modestly induced by STAg (~10-fold), in C3H/OuJ macrophages. In C3H/HeJ macrophages, IP-10 mRNA expression was only induced over baseline by MeXAA, while but-LPS and STAg did not significantly up-regulate IP-10 mRNA expression over levels observed in the medium control. Interestingly, IP-10 mRNA was reduced in LPS-treated C3H/HeJ macrophages. While the induction of IP-10 by MeXAA has been reported (264), the previous failure to detect STAg-induced IP-10 mRNA (263) is most likely due to the less sensitive detection of gene induction by Northern blot analysis used in their previous study, compared to the highly sensitive method of RT-PCR used for this analysis. Of the CC chemokines, RANTES, JE/MCP-1,

MIP-1 $\alpha$ , and MIP-1 $\beta$  all shared similar inducibility profiles in C3H/OuJ and C3H/HeJ macrophages (Figure 8B), illustrating the ability of both MeXAA and STAg to induce this subset of genes almost to the same extent as LPS. MCP-5, the final CC chemokine examined in this panel, proved to be the only chemokine that was poorly induced by STAg in either C3H/OuJ and C3H/HeJ macrophages, nor was it strongly induced by but-LPS in C3H/HeJ macrophages (Figure 8B). Thus, the induction of CXC and CC chemokine mRNA by agents other than LPS in C3H/HeJ macrophages demonstrates that a normal *Lps* (*Tlr4*) gene product is not required for their expression.

**Suppression of LPS-induced chemokine mRNA expression and protein secretion in LPS-tolerant macrophages.** Pretreatment of macrophages with LPS induces a transient state of hyporesponsiveness to subsequent LPS exposure (300). Macrophages rendered "LPS-tolerant" are suppressed in their capacity to produce cytokines like TNF $\alpha$ , IL-1 $\beta$ , IL-12, and IP-10, while the production of other mediators like TNFRII, IL-10, and NO is not suppressed (290, 301, 302). A previous study in our laboratory reported the attenuated production of select chemokines in an *in vivo* model of endotoxin tolerance following septicemia induced by cecal ligation and puncture (159). To extend these observations to an *in vitro* model of LPS-tolerance, C3H/OuJ macrophages were pretreated for 18 h with medium or LPS (100 ng/ml), washed thoroughly, and then stimulated with medium or LPS (10 ng/ml). Detection of mRNA expression for each chemokine was performed at a time that corresponded with their peak levels of LPS-

induction (*i.e.*, 2 h for KC, MIP-1 $\beta$ , and MIP-2; and 6 h for IP-10, MIP-1 $\alpha$ , JE/MCP-1, MCP-5, and RANTES). As shown in Figure 9A and 9B, LPS-pretreatment significantly inhibited the capacity of macrophages to up-regulate the levels of chemokine mRNA expression, except RANTES, following stimulation with LPS ( $p < 0.05$ ). The levels of CXC and CC chemokine mRNA expression in LPS-pretreated, LPS-stimulated (LPS/LPS) macrophages reached only ~5 - 40% of the maximally-induced levels observed in the medium-pretreated, LPS-stimulated (Medium/LPS) macrophages. RANTES mRNA expression in LPS-pretreated, medium-stimulated (LPS/Medium) macrophages remained significantly elevated over baseline at a level equivalent to that observed in medium-pretreated, LPS-stimulated (Medium/LPS) macrophages. This prolonged elevation of LPS-induced RANTES mRNA expression precludes the ability to evaluate the effect of a subsequent exposure to LPS.

To validate and extend the results observed at the level of mRNA expression, we next examined the effect of LPS-tolerance on chemokine protein secretion. In accordance with the mRNA data, LPS-pretreatment significantly inhibited the production of JE/MCP-1, KC, and MIP-2 by macrophages re-stimulated with LPS (Table II). Consistent with previous reports, TNF- $\alpha$ , included as a control, was also significantly inhibited in LPS-pretreated, LPS-stimulated (LPS/LPS) macrophage culture supernatants (290, 300).

**Figure 9.** Suppression of LPS-induced CXC (A) and CC (B) chemokine mRNA expression in LPS-tolerant macrophages. C3H/OuJ macrophages were pretreated for 18 h with LPS (100 ng/ml) or medium, washed thoroughly, and then stimulated with LPS (10 ng/ml) or medium. Detection of mRNA expression for each chemokine was performed at a time that corresponded with their peak levels of LPS-induction (i.e., 2 h for KC, MIP-1 $\beta$ , and MIP-2; and 6 h for IP-10, MIP-1 $\alpha$ , JE/MCP-1, MCP-5, and RANTES). Data are expressed as the mean fold induction  $\pm$  SEM from 4 independent experiments. All chemokine mRNA levels of LPS-pretreated, LPS-challenged macrophages, except RANTES, were significantly lower ( $p < 0.05$ ) than mRNA levels from medium-pretreated, LPS-challenged (Medium/LPS) macrophages.

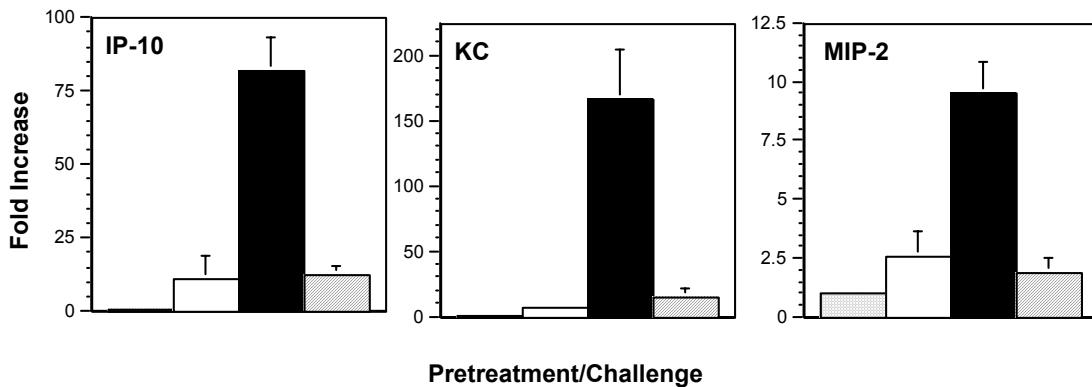
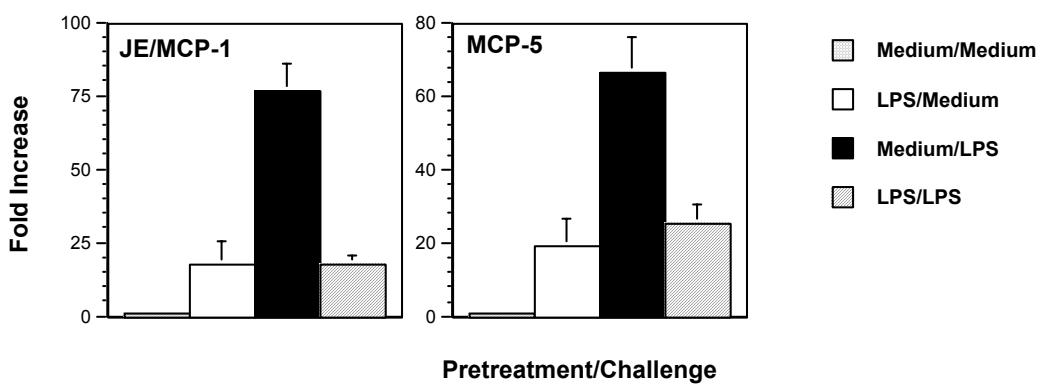
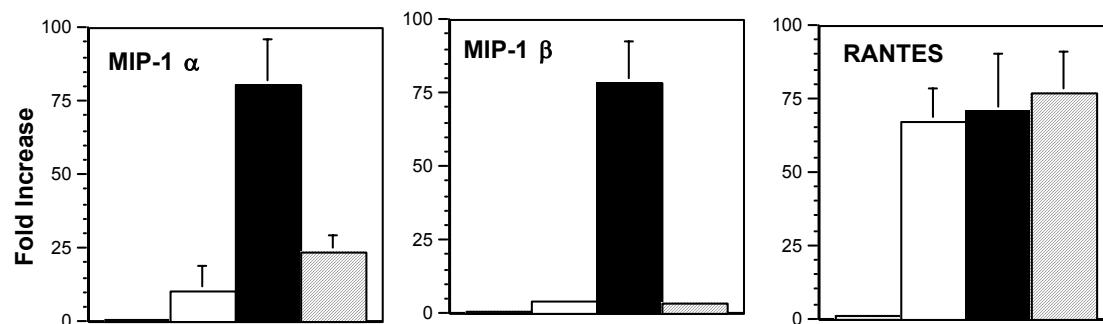
**A. CXC****B. CC**

Table II. Production of JE/MCP-1, KC, MIP-2, and TNF- $\alpha$  in culture supernatants of LPS-tolerized macrophages

Pretreatment/Challenge <sup>a</sup>	JE/MCP-1	KC	MIP-2	TNF- $\alpha$	pg/ml <sup>b</sup>
Medium/Medium	157 $\pm$ 18	7 $\pm$ 4	$\leq$ 15	$\leq$ 70	
LPS/Medium	4,928 $\pm$ 160	1,022 $\pm$ 60	1,075 $\pm$ 117	1,092 $\pm$ 51	
Medium/LPS	14,397 $\pm$ 1,091	19,109 $\pm$ 2,085	10,499 $\pm$ 1,537	22,208 $\pm$ 4241	
LPS/LPS	5,675 $\pm$ 169 <sup>c</sup>	3,728 $\pm$ 499 <sup>c</sup>	590 $\pm$ 166 <sup>c</sup>	3,041 $\pm$ 152 <sup>c</sup>	

<sup>a</sup> Macrophage cultures were pretreated with medium or LPS (100 ng/ml) 18 h prior to re-incubation with medium or LPS (10 ng/ml). Culture supernatants were collected 2 h (MIP-2) or 6 h (JE/MCP-1, KC, and TNF- $\alpha$ ) after treatment.

<sup>b</sup> Data are represented as the mean  $\pm$  SEM from four separate experiments.

<sup>c</sup> Data from LPS/LPS were significantly lower ( $p \leq 0.01$ ) than Medium/LPS.

**IL-10-mediated suppression of LPS-induced chemokine mRNA expression.**

Several studies have demonstrated the ability of IL-10 to down-regulate LPS-inducible mRNA expression of proinflammatory cytokines (IFN- $\gamma$ , IL-1, IL-6, IL-12, and TNF- $\alpha$ ) (292, 303-305) and chemokines (IP-10, KC, MIP-1 $\alpha$  and MIP-1 $\beta$ ) (306-309) in macrophages. To establish whether the expression of different chemokine genes might be variably sensitive to IL-10, as well as determine whether this inhibitory effect could be extended to all the chemokines included in this study, C3H/OuJ peritoneal macrophages were incubated for 6 h with medium alone or 0.1 to 100 ng/ml LPS in the absence or presence of IL-10 (100 U/ml). Figure 10 depicts Southern blots illustrating the suppressive effect of IL-10 on LPS-induced CXC and CC mRNA expression. The ability of IL-10 to down-regulate the LPS-induced mRNA expression proved to be a generalized effect observed for the entire panel of chemokines, although certain chemokine genes, *e.g.*, MIP-1 $\beta$  and RANTES, were less sensitive to inhibition (data not shown). While the inhibitory capacity of IL-10 was evident over a range of LPS doses, the most dramatic reductions (~10 - 80-fold) were observed at lower doses of LPS (0.1 and 1 ng/ml). The inhibitory effects of IL-10 were confirmed at the level of protein production for MIP-2 and JE/MCP-1 (Table III).

**Selective inhibition of LPS-induced chemokine mRNA expression and protein production by tyrphostin AG556.** Protein tyrosine phosphorylation pathways mediate many of the cellular effects of LPS and LPS-induced cytokines like

**Figure 10.** Suppression of LPS-induced chemokine mRNA expression by IL-10.

C3H/OuJ macrophages were cultured for 6 h with medium alone or 0.1 to 100 ng/ml LPS in the absence or presence of IL-10 (100 U/ml). Representative Southern blots of 3 independent experiments for CXC (KC and MIP-2) and CC (JE/MCP-1 and MCP-5) chemokines are shown.

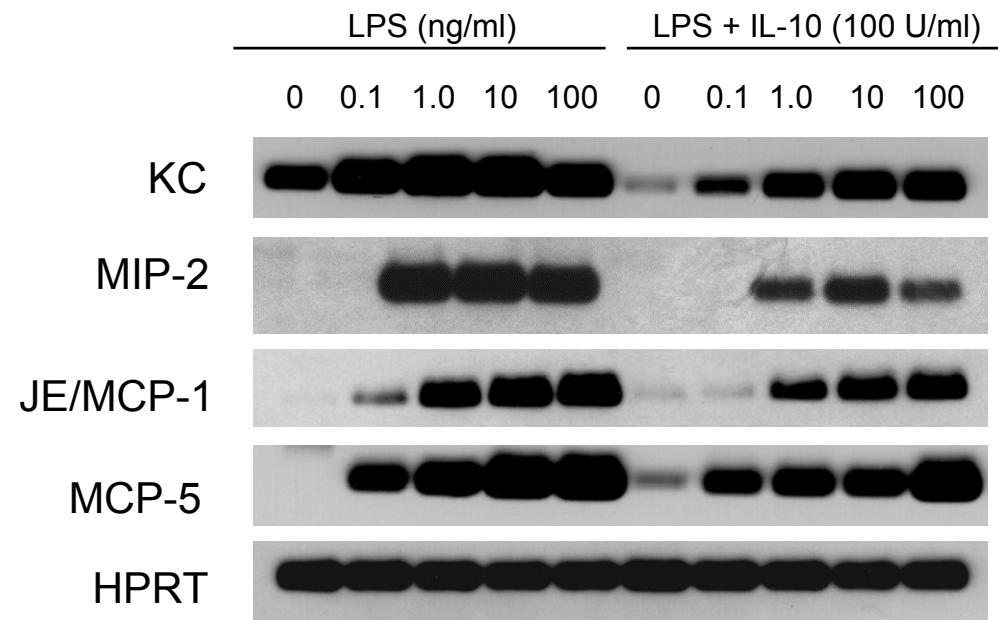


Table III. Effect of IL-10 on the production of JE/MCP-1 and MIP-2 in culture supernatants of LPS-stimulated macrophages

Treatment <sup>a</sup>	pg/ml <sup>b</sup>	
	JE/MCP-1	MIP-2
Medium	< 31	< 16
LPS (1 ng/ml)	6,387 ± 282	50,088 ± 7,251
LPS (1 ng/ml) + IL-10	1,822 ± 404 <sup>c</sup>	10,745 ± 1,890 <sup>c</sup>
LPS (10 ng/ml)	25,912 ± 3,380	89,521 ± 12,416
LPS (10 ng/ml) + IL-10	6,029 ± 289 <sup>c</sup>	29,393 ± 4,117 <sup>c</sup>

<sup>a</sup> Macrophages were treated with medium or LPS (1 or 10 ng/ml) in the absence or presence of IL-10 (100 U/ml). Supernatants were harvested at 6 h and analyzed for chemokine levels by ELISA.

<sup>b</sup> Data are represented as the mean ± SEM from three separate experiments.

<sup>c</sup> The concentration of JE/MCP-1 and MIP-2 in the supernatants of LPS + IL-10-treated macrophages were significantly lower ( $p < 0.05$ ) than those treated with LPS alone.

TNF- $\alpha$  and IL-1 $\beta$  (28, 310-313). While tyrphostins have been shown to inhibit LPS-induced production of TNF- $\alpha$  and NO (314-316), their effect on other inflammatory mediators, like chemokines, has not been evaluated. To determine whether the inhibitory effect of tyrphostin AG556, benzylidene malononitrile derivative, extends to this panel of chemokines, C3H/OuJ peritoneal macrophages were pretreated for 1 h with medium only (not shown), or with medium containing DMSO (0.2%, equivalent concentration in 20  $\mu$ M AG556) or AG556 (20, 10, 5, and 2.5  $\mu$ M), and then incubated for 2 or 6 h with medium or LPS (10 ng/ml). As illustrated in the representative Southern blots in Figure 11, the most profound AG556-mediated inhibition was observed for LPS-induced JE/MCP-1 and MCP-5 mRNA expression, while only modest reductions were observed for RANTES (2 h) and IP-10 (6 h) after pretreatment with the highest dose of AG556 (20  $\mu$ M). Accompanying the substantial reductions in mRNA, production of JE/MCP-1 in supernatants of macrophages pretreated with AG556 (20  $\mu$ M) was significantly inhibited ( $\geq 80\%$ ) at 2 and 6 h after LPS treatment (Table IV). In contrast to its inhibitory effect on select inflammatory mediators, AG556 pretreatment resulted in slightly enhanced LPS-induced MIP-2 mRNA and protein production 6 h after LPS treatment (Figure 11 and Table IV). Pretreatment of macrophages with vehicle only (*e.g.*, 0.2% DMSO) did not result in altered mRNA expression or protein production when compared to medium controls (data not shown).

**Figure 11.** Selective inhibition of LPS-induced chemokine mRNA expression by tyrphostin AG556. C3H/OuJ macrophages were pretreated for 1 h with medium or AG556 (20, 10, 5, and 2.5  $\mu$ M) and then re-incubated for 2 or 6 h with medium or LPS (10 ng/ml). A representative Southern blot of RT-PCR-amplified products is shown (n=3).

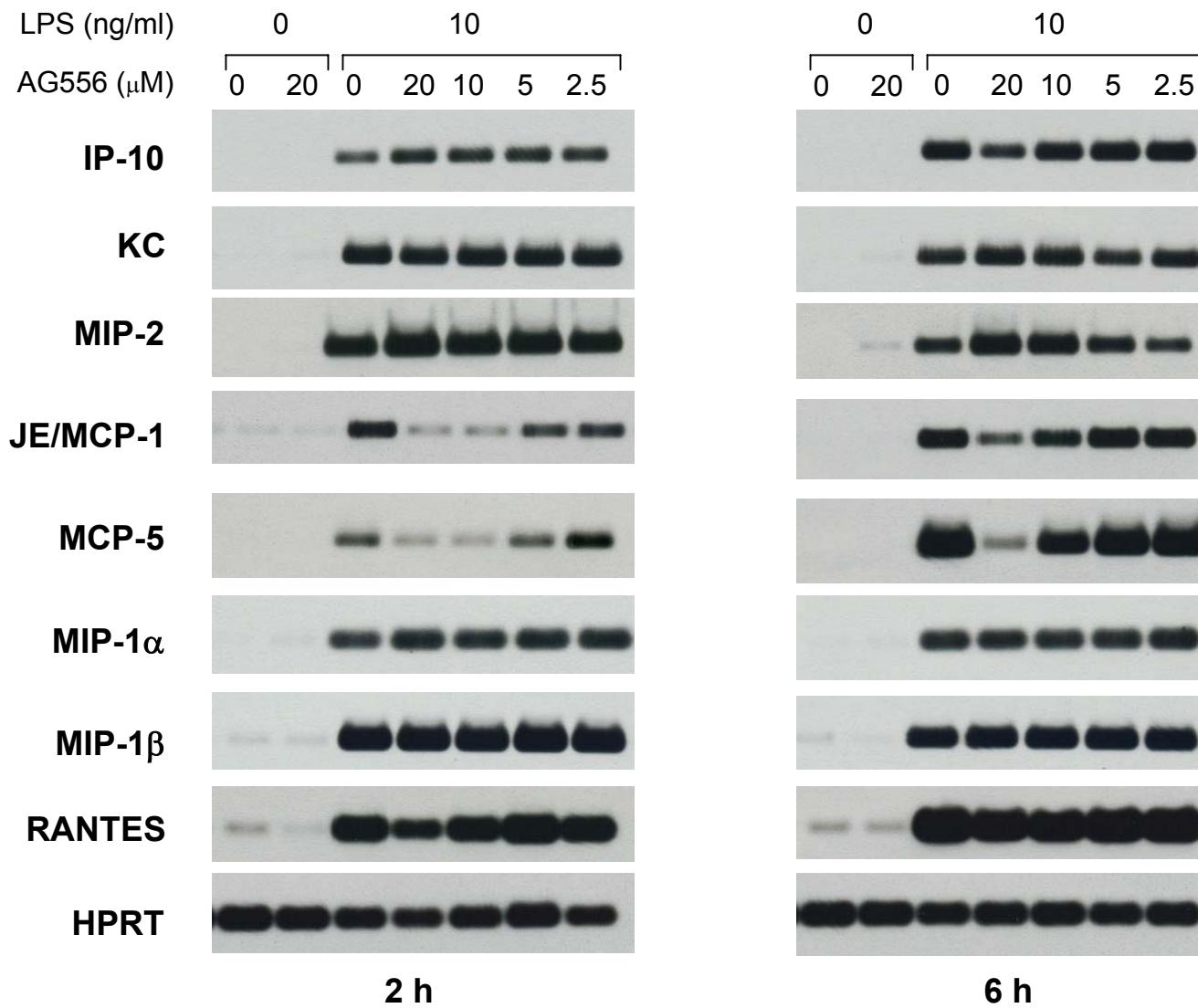


Table IV. Production of JE/MCP-1 and MIP-2 in culture supernatants of LPS-stimulated macrophages pretreated with tyrphostin AG556

Treatment <sup>a</sup>	JE/MCP-1 (pg/ml) <sup>b</sup>		MIP-2 (pg/ml)	
	2 h	6 h	2 h	6 h
Medium	63 ± 5	133 ± 10	17 ± 1	14 ± 4
AG556	21 ± 3	148 ± 28	17 ± 3	79 ± 7
LPS	548 ± 76	10,516 ± 384	8,327 ± 835	24,354 ± 1,401
LPS + AG556	70 ± 15 <sup>c</sup>	2,159 ± 264 <sup>c</sup>	4,404 ± 886 <sup>c</sup>	48,572 ± 8,015 <sup>d</sup>

<sup>a</sup> Macrophage cultures were pretreated with medium or AG556 (20 µM) 1 h prior to re-incubation with medium or LPS (10 ng/ml). Culture supernatants were collected 2 and 6 h after treatment.

<sup>b</sup> Data are represented as the mean ± SEM from three separate experiments.

<sup>c</sup> Data from LPS + AG556 were significantly lower ( $p \leq 0.01$ ) than LPS.

<sup>d</sup> Data from LPS + AG556 were significantly higher ( $p \leq 0.05$ ) than LPS.

**Selective inhibition of LPS-induced chemokine mRNA expression by IFN- $\gamma$ .**

Like LPS, IFN- $\gamma$  serves as a potent activating factor of macrophages. Although IFN- $\gamma$  has been shown to interact synergistically with LPS to induce the mRNA for inflammatory mediators like TNF- $\alpha$  and iNOS (273, 280, 317), it also has been shown to suppress LPS-induced mRNA of other inflammatory mediators like JE/MCP-1, KC, MIP-1 $\alpha$ , and MIP-1 $\beta$  (133, 309). To investigate further the effect of IFN- $\gamma$  on the panel of chemokines in this study, C3H/OuJ peritoneal macrophages were incubated for 6 h with medium alone or LPS (1 ng/ml) in the absence or presence of IFN- $\gamma$  (5 U/ml). Of the eight chemokine genes evaluated, treatment with IFN- $\gamma$  alone was only capable of inducing MCP-5 (~20-25-fold) and IP-10 (~14-fold) mRNA expression (Figure 12). IFN- $\gamma$  inhibited LPS-induced MIP-1 $\beta$  and KC mRNA (~60-70%) and both mRNA and protein for MIP-1 $\alpha$ , JE/MCP-1, and MIP-2 (Figure 12 and Table V). In contrast, mRNA levels of RANTES, MCP-5, and IP-10 were unaffected by simultaneous treatment with LPS and IFN- $\gamma$ .

**Induction of IP-10 and MCP-5 mRNA by LPS or LPS and IFN- $\gamma$  is STAT1-dependent.** IP-10 and MCP-5 were the only chemokine genes that were inducible by either IFN- $\gamma$  or LPS alone. Because IFN- $\gamma$ -mediated responses use activated STAT1 as a major transactivating factor, we sought to evaluate the role of STAT1 in LPS-induced chemokine gene expression. Thus, macrophages derived from wild-type (STAT1 $^{+/+}$ ) and STAT1 knockout (STAT1 $^{-/-}$ ) mice were

**Figure 12.** Selective inhibition of IFN- $\gamma$  on LPS-induced CC (A) and CXC (B) chemokine mRNA expression. C3H/OuJ macrophages were cultured for 6 h with medium alone or LPS (1 ng/ml) in the absence or presence of IFN- $\gamma$  (5 U/ml). Data are expressed as the mean-fold induction  $\pm$  SEM from 3 experiments. An asterisk with the percent reduction from LPS-stimulated values is included only when significantly different ( $p < 0.05$ ).

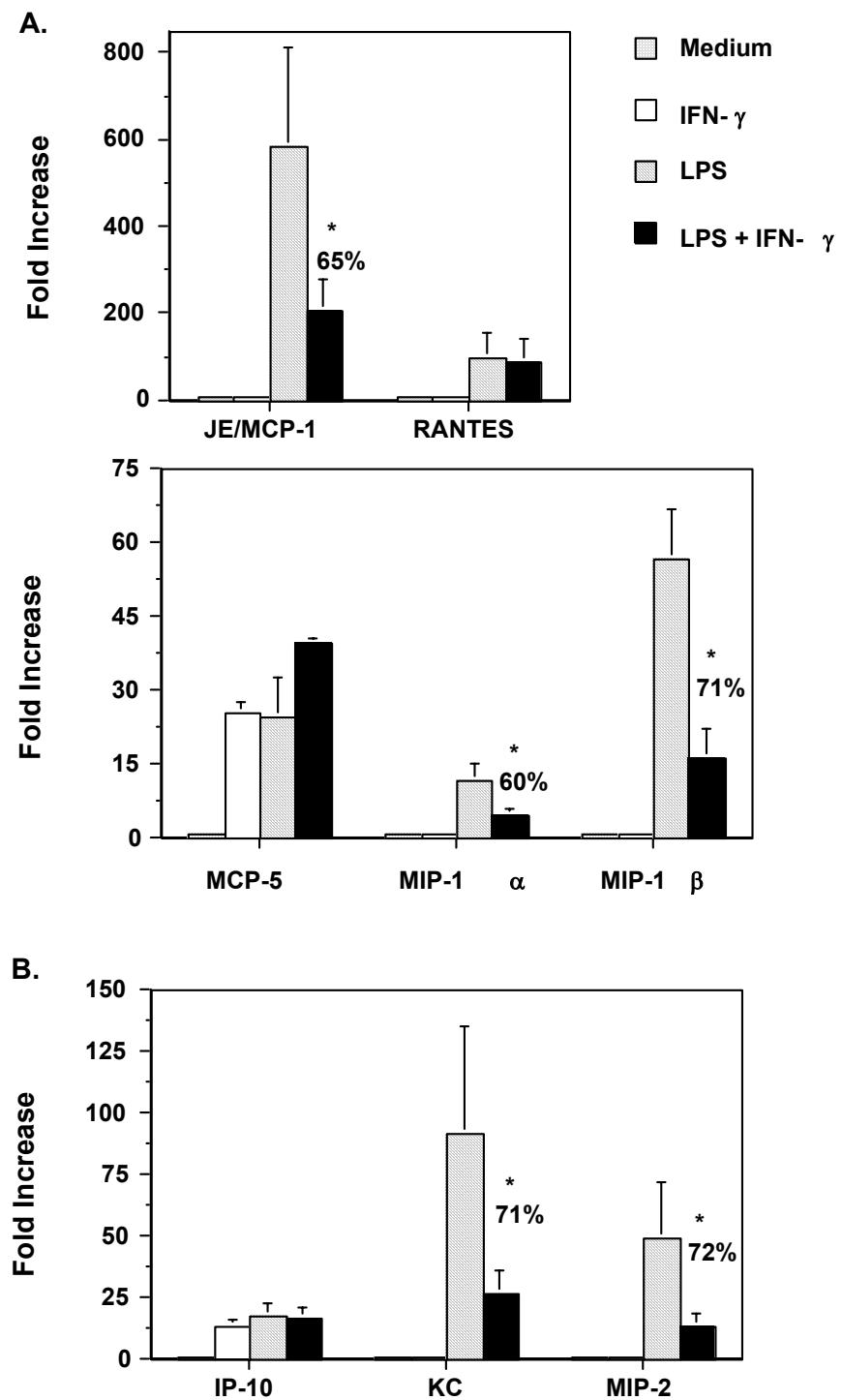


Table V. Effect of IFN- $\gamma$  on the production of JE/MCP-1, MIP-1 $\alpha$ , and MIP-2 in culture supernatants of LPS-stimulated macrophages

Treatment <sup>a</sup>	JE (pg/ml)		MIP-1 $\alpha$ (pg/ml)		MIP-2 (pg/ml)	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2
Medium	75	28	< 9.4	< 9.4	58	59
IFN- $\gamma$	89	33	< 9.4	< 9.4	58	58
LPS	4,114	2,260	4,428	3,903	12,028	11,102
LPS + IFN- $\gamma$ <sup>b</sup>	2,000	1,448	2,938	2,903	6,567	6,558

<sup>a</sup> Macrophages were treated with medium or LPS (1 ng/ml) in the absence or presence of IFN- $\gamma$  (5 U/ml). Supernatants were harvested at 6 h and analyzed for chemokine levels by ELISA.

<sup>b</sup> The concentration of JE/MCP-1, MIP-1 $\alpha$ , and MIP-2 in the supernatants of LPS + IFN- $\gamma$ -treated macrophages were significantly lower ( $p < 0.05$ ) than those treated with LPS alone ( $n = 3-6$ ).

compared for their ability to respond to LPS or LPS and IFN- $\gamma$ . Of the eight chemokine genes examined, only LPS-induced IP-10 and MCP-5 mRNA expression were markedly abrogated in macrophages from STAT1<sup>-/-</sup> mice (Figure 13). These data suggest a key role for STAT1 in the induction of IP-10 and MCP-5 by LPS.

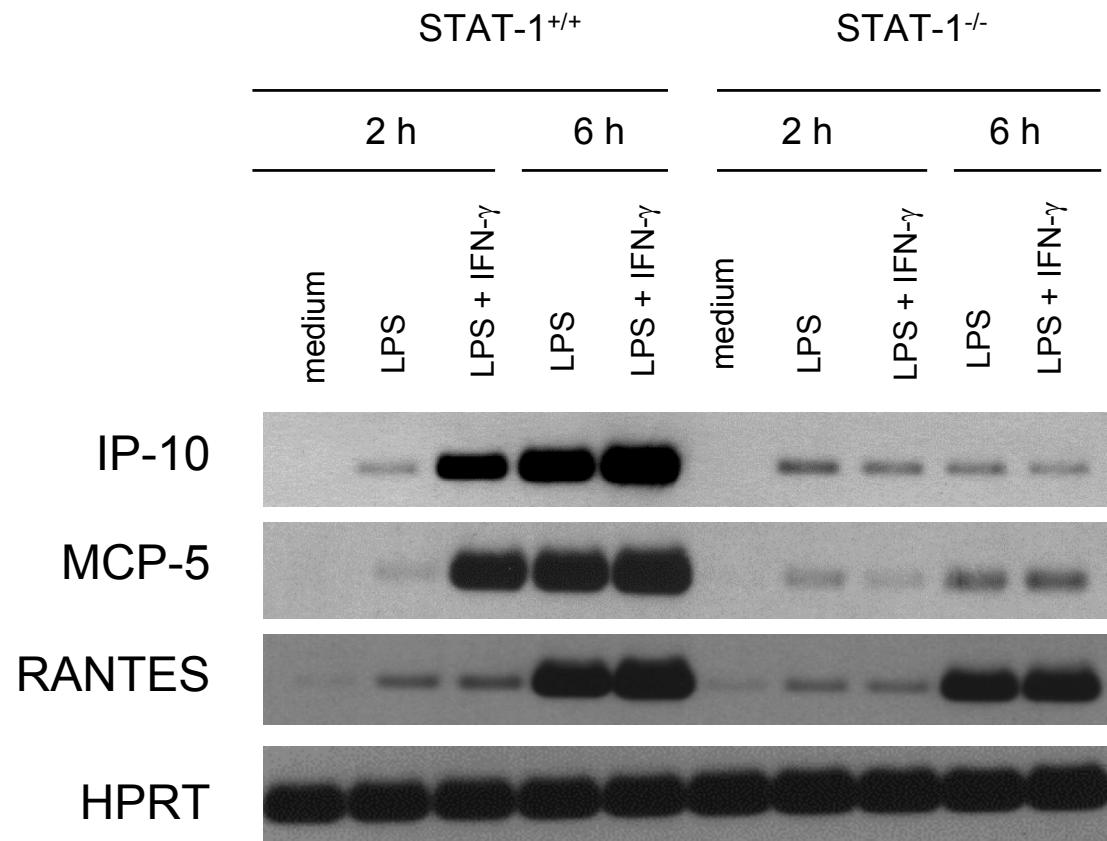
**IFN- $\gamma$ -mediated inhibition of LPS-induced KC mRNA is dependent on IRF-2.**

The availability of mice deficient in the IFN-responsive transcription factor genes, IRF-1 and IRF-2, provided an opportunity to assess the role of these factors in the suppressive activity of IFN- $\gamma$  on selected chemokine genes.

Peritoneal macrophages from IRF-1<sup>+/+</sup> or IRF-1<sup>-/-</sup> or IRF-2<sup>+/+</sup> or IRF-2<sup>-/-</sup> mice were incubated for 6 h with medium alone or LPS (1 ng/ml) in the absence or presence of IFN- $\gamma$  (5 U/ml). Induction of chemokine mRNA by LPS was neither IRF-1 nor IRF-2-dependent (data not shown). However, the ability of IFN- $\gamma$  to suppress KC gene expression was entirely lost in macrophages from IRF-2<sup>-/-</sup> mice, while suppression of the other IFN- $\gamma$  sensitive chemokine genes (*e.g.*, JE/MCP-1, MIP-2, MIP-1 $\alpha$ , and MIP1 $\beta$ ) was unaffected by deletion of either gene (Figure 14).

Specifically, IFN- $\gamma$  inhibited LPS-induced KC mRNA expression (~72-84%) in IRF-2<sup>+/+</sup> macrophages, while KC mRNA levels in IRF-2<sup>-/-</sup> macrophages were reduced only slightly. This de-repression of LPS-induced KC mRNA by IFN- $\gamma$  observed in IRF-2<sup>-/-</sup> macrophages was accompanied by elevated levels of KC protein in culture supernatants (Table VI). In contrast, the IFN- $\gamma$ -mediated

**Figure 13.** The effect of Stat1 on the induction of LPS- and LPS plus IFN- $\gamma$ -induced mRNA expression. Macrophages from Stat1<sup>+/+</sup> and Stat1<sup>-/-</sup> mice were cultured for 2 and 6 h with medium alone or LPS (1 ng/ml) in the absence or presence of IFN- $\gamma$  (5 U/ml). Total RNA from control and treated Stat1<sup>+/+</sup> and Stat1<sup>-/-</sup> macrophages was kindly provided by Thomas A. Hamilton and Jennifer Major (Cleveland Clinic Foundation, Cleveland, OH). Representative Southern blots of one of two independent experiments are shown.



**Figure 14.** The effect of IRF-2 on the IFN- $\gamma$ -mediated inhibition of LPS-induced mRNA expression. Macrophages from IRF-2<sup>+/+</sup> and IRF-2<sup>-/-</sup> mice were cultured for 6 h with medium alone or LPS (1 ng/ml) in the absence or presence of IFN- $\gamma$  (5 U/ml). Representative graphs of one of three independent experiments are shown. The percent reduction from LPS-stimulated values is included.

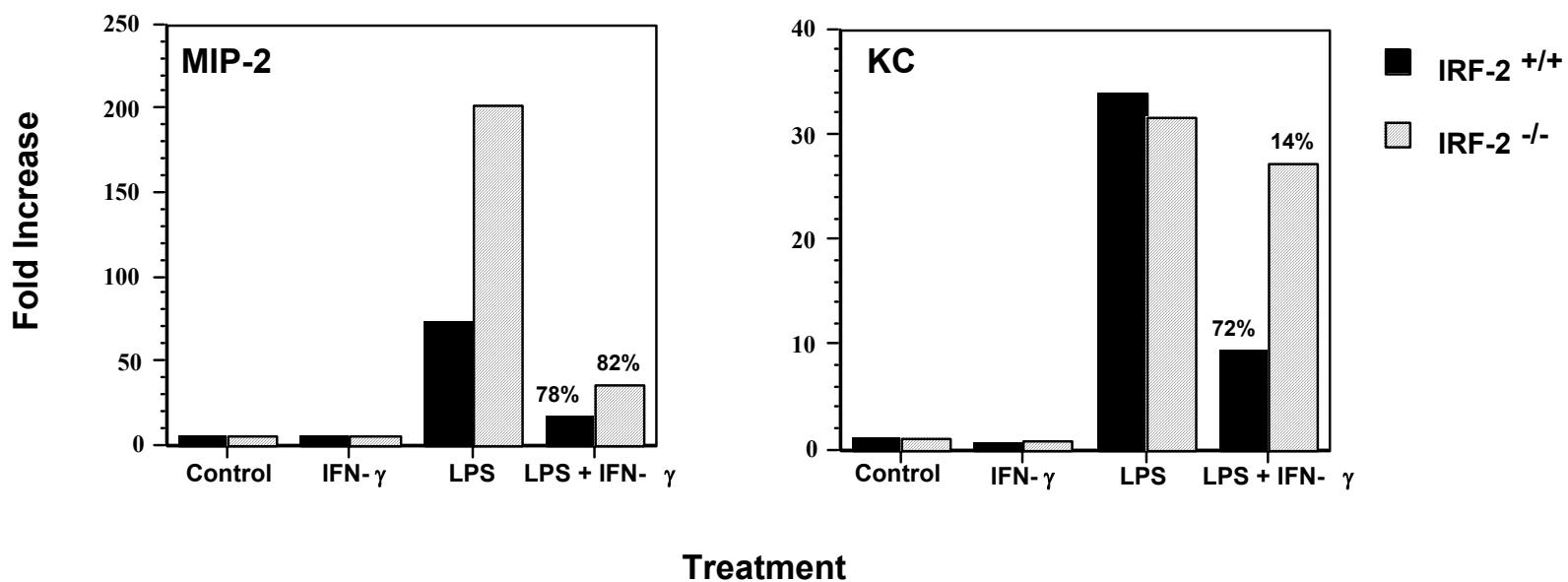


Table VI. Effect of IFN- $\gamma$  on the production of KC in culture supernatants of LPS-stimulated macrophages of IRF-2<sup>+/+</sup> and IRF-2<sup>-/-</sup> mice

Treatment <sup>a</sup>	KC (pg/ml)			
	Expt. 1		Expt. 2	
	IRF-2 <sup>+/+</sup>	IRF-2 <sup>-/-</sup>	IRF-2 <sup>+/+</sup>	IRF-2 <sup>-/-</sup>
Medium	< 32	< 32	< 32	< 32
IFN- $\gamma$	< 32	< 32	< 32	< 32
LPS	3,596	3,089	5,538	5,253
LPS + IFN- $\gamma$	659 (82%) <sup>b</sup>	1,873 (39%)	948 (83%)	2,948 (43%)

<sup>a</sup> Macrophages were treated with medium or LPS (1 ng/ml) in the absence or presence of IFN- $\gamma$  (5 U/ml). Supernatants were harvested at 6 h and analyzed for KC levels by ELISA.

<sup>b</sup> Percent reduction from LPS-stimulated value (100%) is included in parenthesis.

inhibition of LPS-induced JE/MCP-1, MIP-2, MIP-1 $\alpha$ , and MIP-1 $\beta$  mRNA was not reversed in IRF-1 $^{-/-}$  or IRF-2 $^{-/-}$  macrophages (Figure 14 and data not shown). These data support a role for IRF-2 in IFN- $\gamma$ -mediated inhibition of LPS-induced KC mRNA.

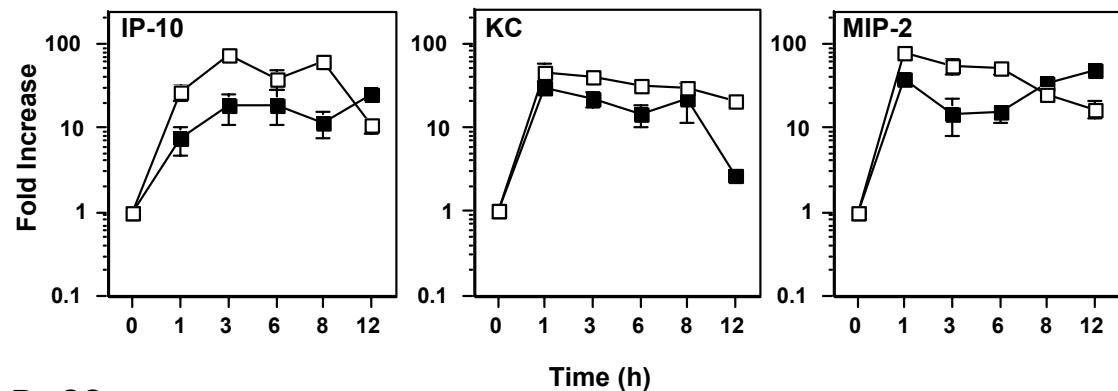
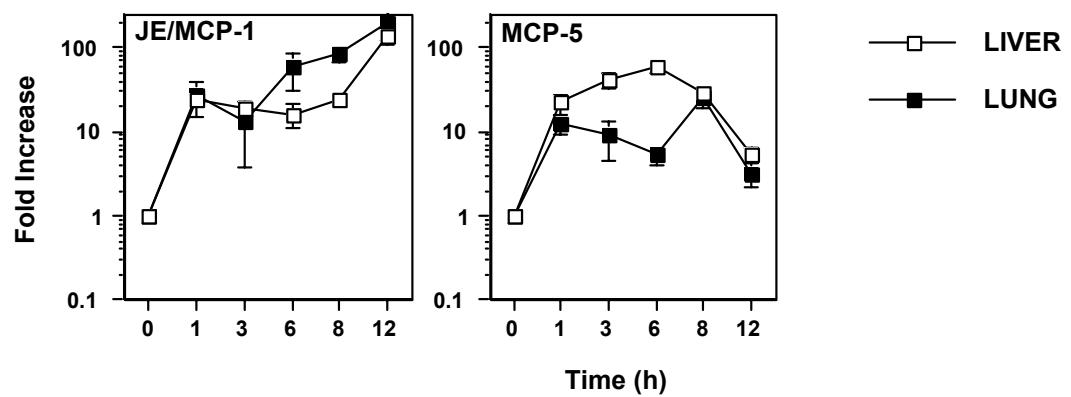
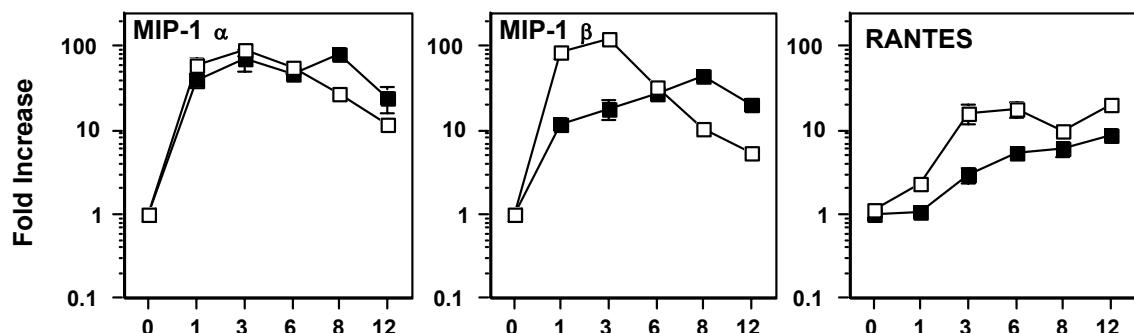
### **B. *In Vivo* Analysis of LPS-Induced Chemokine Expression**

While the *in vitro* studies focused on the induction of chemokines as a result of the interaction of LPS with macrophages, the *in vivo* studies extend the analysis to encompass the broad array of cell types that participate in the physiologic response to LPS in the host. Multiple cell types (*i.e.*, leukocytes, endothelial cells, epithelial cells, fibroblasts, hepatocytes, dendritic cells, etc.) have been shown to produce chemokines in response to LPS {see reviews (83-87)}. Consequently, measurements in serum and organs represent a composite of responding cell types that have differential induction kinetics and sensitivities to LPS. Therefore, to extend the observations *in vitro*, we conducted a systematic and comprehensive evaluation of LPS-induced chemokine regulation *in vivo* to further our understanding of the complexities of chemokine regulation in response to LPS.

**Kinetic Analysis of CXC and CC chemokine mRNA expression in tissue after administration of LPS.** Systemic exposure with LPS initiates a highly coordinated recruitment of neutrophils and monocytes into the liver and lung (71, 173, 318, 319). To gain insights into the pattern of tissue-specific chemokine

expression, we examined the temporal induction of pulmonary and hepatic chemokine mRNA expression in a murine model of endotoxemia. For these studies, C57BL/6J mice were injected i.p. with 25 µg LPS and chemokine mRNA was analyzed in the liver and lung at 1, 3, 6, 8, and 12 h after administration of LPS. Variations in the basal expression of these genes in the liver and lung, as evidenced by the amplification cycle numbers required to detect the corresponding PCR product (see Table I), precludes direct comparison between these organs. As illustrated in Figure 15, hepatic IP-10, KC, MIP-2, MIP-1 $\alpha$ , MIP-1 $\beta$ , JE/MCP-1, and MCP-5 were rapidly induced (~20-80-fold) by 1 h after administration of LPS. Hepatic IP-10, KC, MIP-2, MIP-1 $\alpha$ , and MIP-1 $\beta$  mRNA reached peak levels (~50-100-fold) by 1 to 3 h after LPS. A slower induction of peak mRNA levels in the liver was observed for MCP-5 (6 h; ~60-fold) and JE/MCP-1 (12 h; ~100-fold). Of the chemokine genes examined in the liver, only the induction of RANTES mRNA was delayed in onset (3 h), and was modestly induced (~20-fold). The kinetics of LPS-induced chemokine mRNA expression in the lung generally paralleled the inductive pattern observed in the liver. One notable difference was the reduced levels of pulmonary chemokine mRNA expression observed for some genes (*e.g.*, MIP-1 $\beta$  and MCP-5). In general, steady-state chemokine mRNA levels in the liver and lung remained elevated above baseline for 12 h with only gradual declines from peak levels. Only pulmonary and hepatic MCP-5 mRNA and pulmonary KC mRNA had returned to basal levels by 12 h after LPS.

**Figure 15.** Kinetics of CXC (A) and CC (B) chemokine mRNA expression in the lung and liver of C57BL/6J mice after LPS challenge. Mice were injected i.p. with either saline or LPS (25 µg) and lung and liver were harvested at the indicated times for the detection of relative changes in chemokine mRNA expression by RT-PCR. Data are expressed as the mean ± SEM from 4-8 individual mice. The means are expressed relative to the response of untreated mice ( $t = 0$ ), which are assigned a value of 1. When not visible, error bars are smaller than the symbol.

**A. CXC****B. CC**

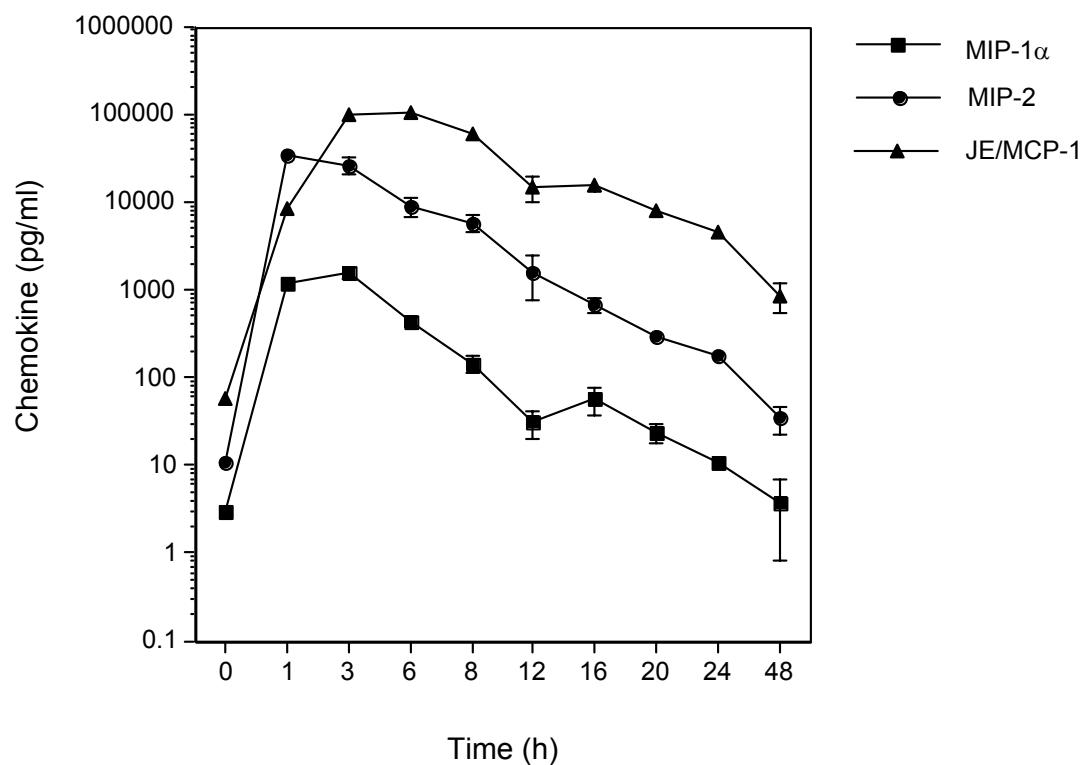
**Kinetic analysis of chemokine levels in the serum after administration of LPS.**

To determine whether the profound increases in tissue chemokine mRNA expression following administration of LPS were associated with a concomitant elevation of circulating chemokines, we examined the kinetics of one CXC (MIP-2) and two CC (JE/MCP-1 and MIP-1 $\alpha$ ) chemokines following administration of LPS. Sera were collected from C57BL/6J mice at multiple time points after i.p. injection with 25  $\mu$ g LPS. As shown in Figure 16, MIP-2, JE/MCP-1, and MIP-1 $\alpha$  were all rapidly and substantially elevated in the serum within 1 h of LPS administration. Maximal concentrations of MIP-2 and MIP-1 $\alpha$  were observed 1 to 3 h after LPS (~30,000 and ~1,500 pg/ml, respectively). In contrast, maximal levels of JE/MCP-1 (~100,000 pg/ml) were observed 3 to 6 h after LPS. Not only was the magnitude of JE/MCP-1 production the most profound, but also its sustained elevation over baseline 24 h after LPS when compared to the more rapidly declining levels of MIP-1 $\alpha$  and MIP-2.

**Effect of macrophage depletion on chemokine mRNA expression in the liver.**

An examination of cytokine gene expression in LPS-stimulated, macrophage-depleted mice, revealed that hepatic macrophages (or Kupffer cells) are the major cellular source of several cytokines (*i.e.*, IL-1 $\beta$ , IL-6, IL-10, IL-12p40, and TNF- $\alpha$ ) in the liver (68). Previous studies by our laboratory and others have demonstrated that i.v. administration of Cl<sub>2</sub>MBP-liposomes selectively depletes macrophages from the liver without altering the number and/or function of

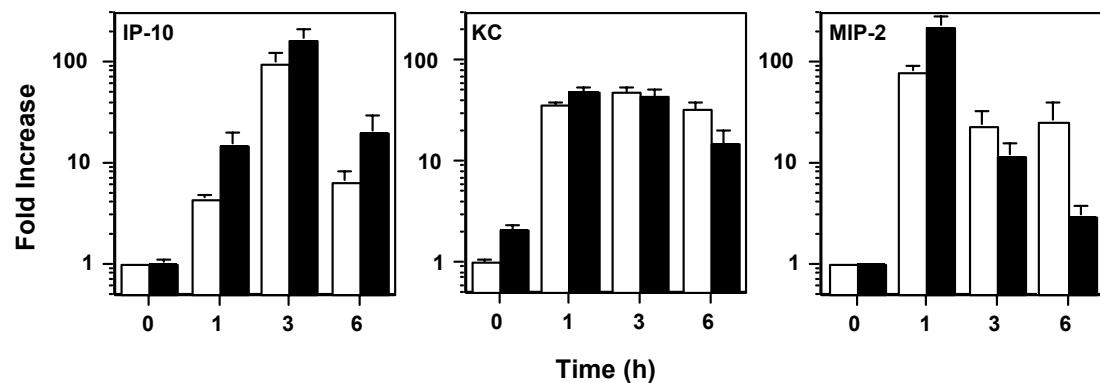
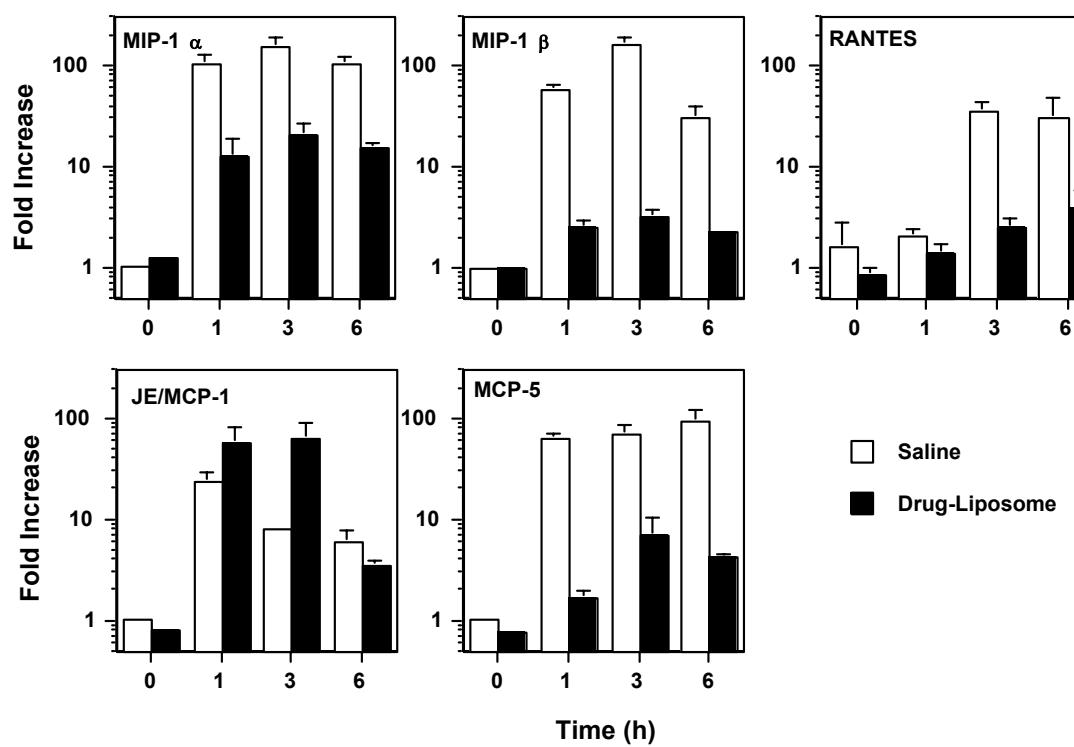
**Figure 16.** Production of MIP-2, MIP-1 $\alpha$ , and JE/MCP-1 in the serum of C57BL/6J mice after LPS challenge. Mice were injected i.p. with either saline ( $t = 0$ ) or LPS (25  $\mu$ g) and serum was collected for chemokine protein production by ELISA. Data are expressed as the mean  $\pm$  SEM from 4 individual mice. When not visible, error bars are smaller than the symbol.



neutrophils, endothelial cells, dendritic cells, or B and T cells (68, 266, 272, 320).

To evaluate the contribution of hepatic macrophages to the *in vivo* induction of chemokine mRNA, C57BL/6J mice were injected i.v. with 0.2 ml of saline, Cl<sub>2</sub>MBP-, or PBS-liposomes 2 days prior to i.p. injection with 25 µg of LPS. Liver specimens were harvested from mice at 1, 3, and 6 h after LPS, then total RNA was extracted and analyzed for gene induction by RT-PCR. Figure 17 illustrates that the induction of MIP-1 $\alpha$ , MIP-1 $\beta$ , RANTES, and MCP-5 mRNA by LPS was reduced by 80-97% in the liver of macrophage-depleted mice when compared to saline-pretreated LPS-injected control mice, indicating that macrophages are largely responsible for the expression of these genes in response to LPS. Moreover, these dramatic reductions in hepatic mRNA levels were detected as early as 1-3 h after LPS and mRNA levels remained reduced for 6 h. In contrast, the induction of MIP-2, IP-10, and JE/MCP-1 mRNA by LPS in the livers of macrophage-depleted mice were slightly elevated at 1-3 h after LPS when compared to control mice. By 6 h after LPS, however, the level of MIP-2 mRNA from macrophage-depleted mice was reduced by ~90%. The induction of KC mRNA by LPS in both treatment groups remained largely unaffected until 6 h after LPS when a slight decrease in mRNA from macrophage-depleted mice was detected. Similar increases in chemokine mRNA expression was observed in PBS-liposome-pretreated, LPS-injected mice when compared to saline-pretreated, LPS-injected mice (data not shown). Since i.v. administration of Cl<sub>2</sub>MBP-

**Figure 17.** Kinetics of CXC (A) and CC (B) chemokine mRNA in the livers of control (open bars) and Kupffer cell-depleted (solid bars) C57BL/6J mice after LPS (25 µg) challenge. Data are expressed as the mean ± SEM from a total of 9 individual mice from two independent experiments. The means are expressed relative to the response of untreated mice, which are assigned a value of 1. When not visible, error bars are smaller than the symbol. An asterisk indicates that mRNA levels from livers of Kupffer cell-depleted mice are significantly different ( $p < 0.05$ ) from mRNA levels from livers of control mice.

**A. CXC****B. CC**

liposomes does not deplete pulmonary macrophages (266), chemokine mRNA expression in the lung was not examined.

### C. Analysis of the Role of KC in a Murine Model of LPS-Induced Lethality

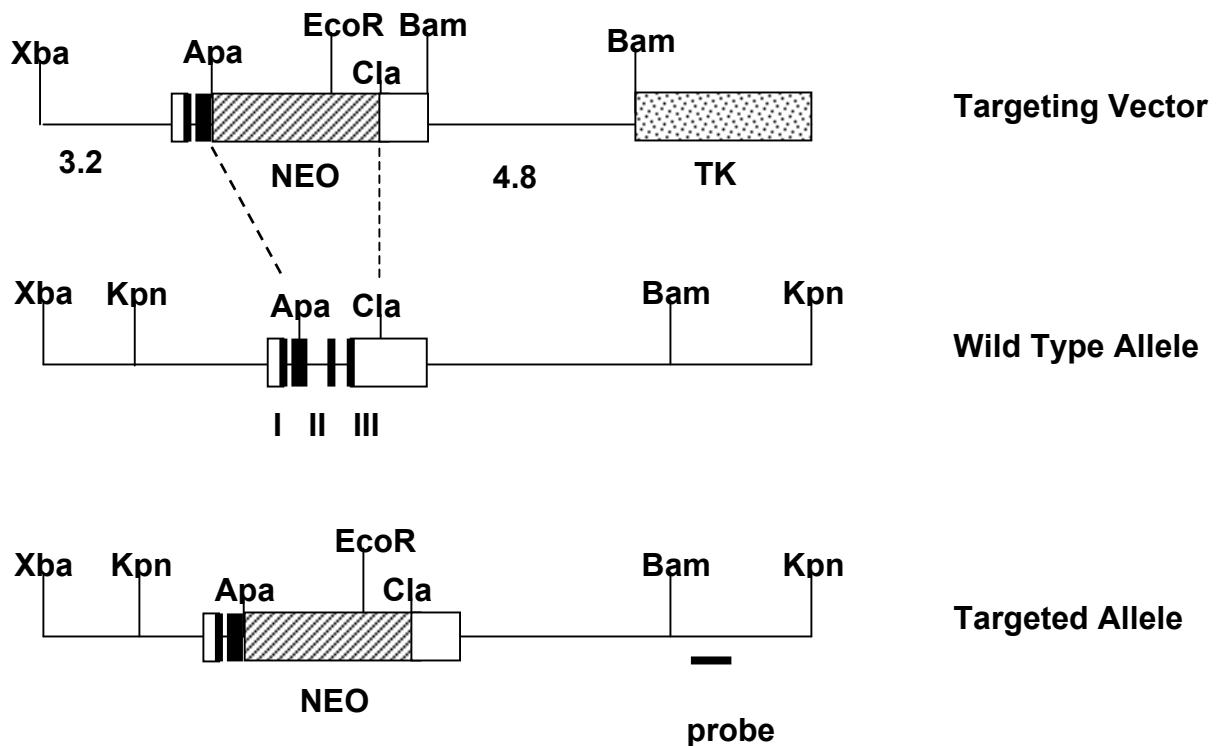
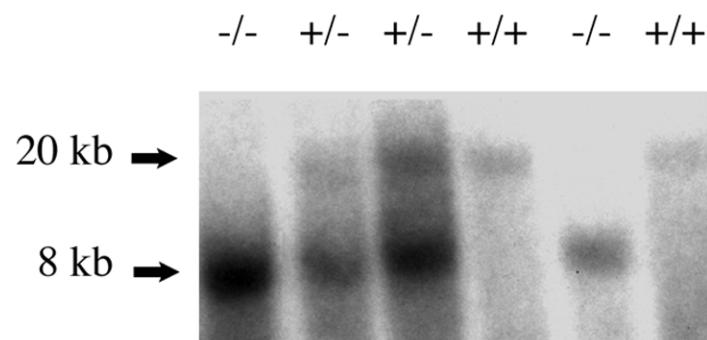
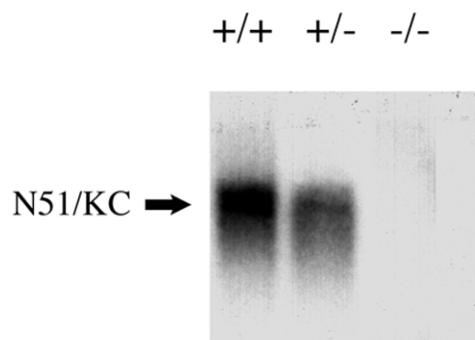
As already stated in the Introduction, the CXC chemokine, KC, is a potent chemoattractant of neutrophils that is dramatically up-regulated *in vitro* and *in vivo* in response to LPS (117, 133, 253). Besides KC, the other known neutrophil chemoattractants in mice are the ELR<sup>+</sup> CXC chemokines, MIP-2, LIX, and GCP-2, and the CC chemokine, MIP-1 $\alpha$  (71, 118, 123, 321, 322). Like KC, these neutrophil chemoattractants are also induced by LPS, further underscoring the potential for functional redundancy.

Because systemic exposure to LPS initiates a rapid recruitment of inflammatory leukocytes, like neutrophils and monocytes, into specific host tissue, the question of whether any one neutrophil chemoattractant is essential to the process remains to be elucidated. The contribution of an individual chemokine in this process is particularly relevant since the influx and activation of inflammatory leukocytes, coupled with the overproduction of inflammatory mediators, are believed to underlie the tissue damage that precedes multiple organ failure and death. The apparent functional redundancy exhibited among chemokines legitimizes the question of whether a single chemokine could play a critical role in the inflammatory response to LPS. One approach for defining the individual role of a chemokine is through the use of gene "knockout" mice in which a single gene of interest is targeted for disruption. Unlike antibody

neutralization studies that encounter problems with specificity, incomplete blockage, and secondary effects of exogenous antibody, studies involving knockout mice afford complete genetic and functional deletion of the gene of interest. The availability of KC knockout mice allowed us to evaluate the individual contribution of KC in a model of LPS-induced lethality. Therefore, to test the hypothesis that KC plays a unique role in the inflammatory response to LPS, we evaluated mice with a targeted disruption in KC in a model of LPS-induced lethality.

**Targeted disruption of the mouse N51/KC gene.** The targeting strategy used to disrupt N51/KC gene expression was developed by Sergio Lira and is depicted in Figure 18A. An ApaI-Clal fragment containing exons II (partial), III, and IV was deleted and replaced with a neomycin-resistance cassette by homologous recombination in 129/Sv-derived ES cells. Male chimeric mice, generated by injecting correctly targeted ES clones into C57BL/6J blastocysts, were bred to C57BL/6J females to obtain germline transmission of the mutated allele. Matings between F<sub>1</sub> heterozygotes yielded the expected Mendelian ratio (1:2:1) of wild-type, heterozygous, and null mice, respectively, at the KC locus as confirmed by Southern blot analysis (Figure 18B). KC<sup>-/-</sup> mice were inbred, resulting in a mixed C57BL/6 and 129/Sv background. Therefore, C57BL/6J × 129/Sv F<sub>1</sub> mice were used as KC<sup>+/+</sup> controls. The absence of KC mRNA expression in KC<sup>-/-</sup> mice was confirmed by Northern blot analysis (Figure 18C)

**Figure 18.** Targeted disruption of the mouse N51/KC gene. (A) The targeting vector, constructed as described in the *Materials and Methods*, is illustrated at the top of panel A. A partial restriction map of the wild-type N51/KC gene locus, center, depicts the 4 coding exons (black boxes) and the 5'- and 3'-untranslated regions (UTR) (white boxes). The predicted structure of the targeted allele after homologous recombination is shown on the bottom. The 0.5 kb fragment used as a probe in Southern blot analysis is indicated by a black bar on the targeted allele. (B) Genomic DNA from F2 progeny of heterozygous matings was digested with KpnI and EcoRV and analyzed by Southern blot using the probe depicted in A. The wild-type (20 kb) and targeted (8 kb) fragments are indicated by the arrows. The genotype of each mouse is shown above the lane (+/+, wild-type; +/-, heterozygous; and -/-, homozygous mutant for N51/KC). (C) Northern blot analysis of total RNA (10 µg/lane) isolated from livers of mice injected i.p. with 100 µg LPS. The blot was probed with a 0.3 kb ApaI-ClaI N51 cDNA fragment. The genotyping, Southern blot analysis, and Northern blot analysis was performed at Bristol-Myers Squibb.

**A.****B.****C.**

and by RT-PCR (Figure 21A and B). Additionally, the absence of KC protein production was verified by Western blot analysis (S. Lira, Schering-Plough Research Institute, Kenilworth, NJ, personal communication) and by ELISA (Figure 20C). Normal cell populations from bone marrow, lymph nodes, spleen, and thymus were evaluated at Bristol-Myers Squibb by flow cytometry with markers for neutrophils (GR-1), monocytes/macrophages (Mac-1), and B (B220, sIgM) and T (CD3) lymphocytes. No significant alterations were observed in the distribution of cell populations between wild-type and KC<sup>-/-</sup> mice (Table VII). However, slightly elevated numbers of neutrophils and B lymphocytes were noted in the bone marrow and lymph nodes, respectively.

**Reduction of LPS-induced mortality in KC<sup>-/-</sup> mice.** To assess the contribution of KC in LPS-induced lethality, KC<sup>+/+</sup> ( $n = 22$ ) and KC<sup>-/-</sup> mice ( $n = 23$ ) were injected i.p. with a lethal dose of LPS (35 mg/kg) and survival was monitored daily for 7 days. Within 1 hr after administration of LPS, both KC<sup>+/+</sup> and KC<sup>-/-</sup> mice developed symptoms associated with endotoxicity to include lethargy, piloerection, and shivering (a sign of fever), followed by diarrhea and watery eyes (a sign of enhanced vasopermeability) several hours later. Despite comparable symptomatology, only 26% of KC<sup>-/-</sup> mice died 24 hr after administration of LPS, compared to 91% of KC<sup>+/+</sup> mice (Figure 19). By 48 hr after LPS administration, 100% of KC<sup>+/+</sup> mice had succumbed to LPS-induced lethality. In contrast, the mortality of KC<sup>-/-</sup> mice progressed at a slower rate and to a lesser extent, with only 52% mortality after 7 days. Furthermore, the

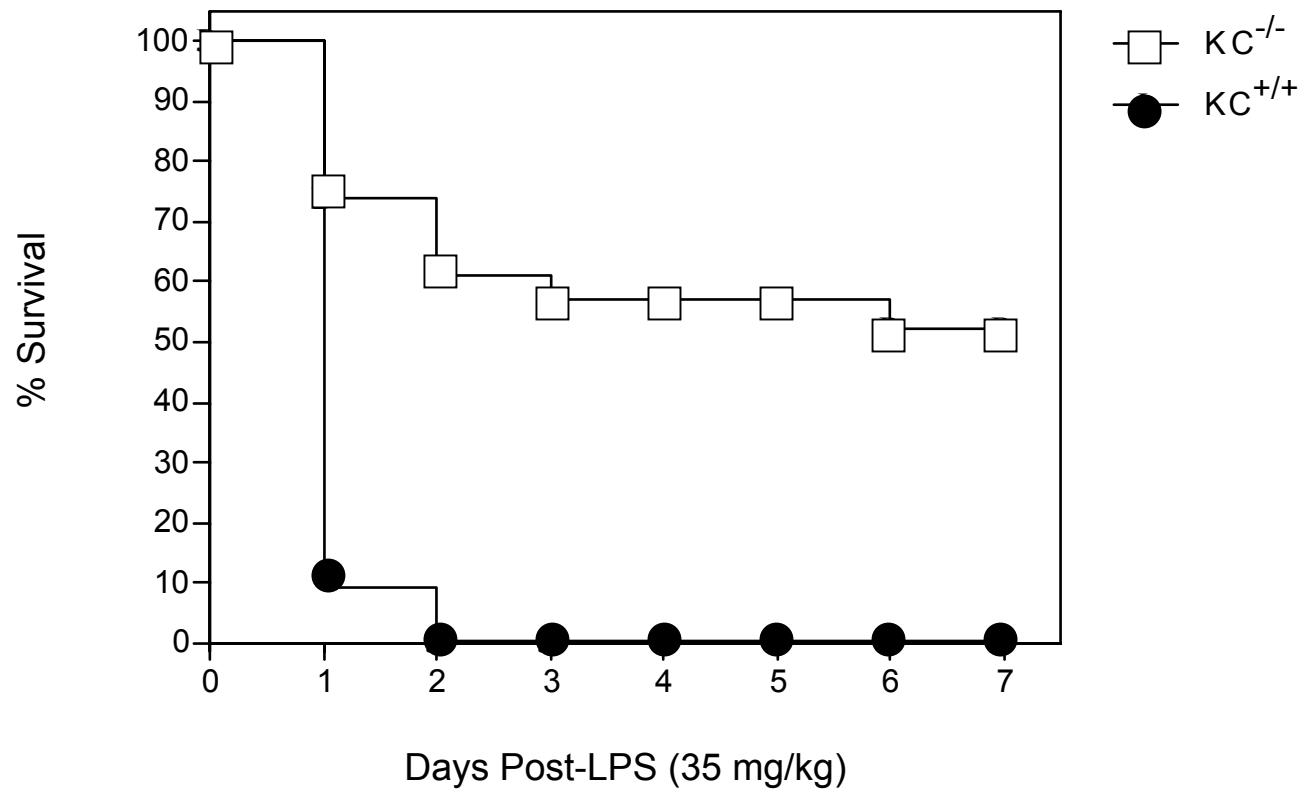
Table VII. Distribution of phenotypic markers on bone marrow, lymph node, and spleen cells from KC<sup>+/+</sup> and KC<sup>-/-</sup> mice

Antibody	% Positive <sup>a</sup>					
	Bone Marrow		Lymph Node		Spleen	
	KC <sup>+/+</sup>	KC <sup>-/-</sup>	KC <sup>+/+</sup>	KC <sup>-/-</sup>	KC <sup>+/+</sup>	KC <sup>-/-</sup>
Gr-1	29.3 ± 4.0	48.8 ± 8.8	26.7 ± 4.8	28.1 ± 4.6	23.7 ± 1.4	30.0 ± 1.2
Mac-1	27.8 ± 3.6	46.4 ± 8.8	15.6 ± 3.9	21.4 ± 8.6	16.2 ± 3.4	25.6 ± 2.1
Gr-1 + Mac-1	31.8 ± 3.6	46.5 ± 7.9	13.7 ± 3.0	19.0 ± 4.8	15.1 ± 3.3	23.7 ± 1.0
M7-4 (LFA-1)	31.0 ± 2.0	50.1 ± 7.9	ND <sup>b</sup>	ND	14.2 ± 2.3	19.1 ± 1.5
B220	48.3 ± 7.7	30.8 ± 7.3	36.2 ± 8.7	55.2 ± 8.8	53.3 ± 4.2	61.9 ± 7.0
IgM	13.1 ± 2.8	9.5 ± 1.1	29.2 ± 5.7	46.8 ± 9.0	48.3 ± 4.2	57.9 ± 3.9
B220 + IgM	20.1 ± 3.7	13.7 ± 2.7	29.5 ± 5.7	47.6 ± 9.0	43.9 ± 4.5	51.4 ± 6.0

<sup>a</sup> Data are represented as the mean ± standard deviation from 3 mice.

<sup>b</sup>Not done.

**Figure 19.** Reduction of LPS-induced mortality in KC<sup>-/-</sup> mice. A lethal dose of LPS (35 mg/kg) was administered i.p. to KC<sup>+/+</sup> mice (n = 22) and age-matched KC<sup>-/-</sup> mice (n = 23). Mortality was monitored daily for 7 d. The cumulative results were derived from two independent experiments which each showed very comparable outcomes.



surviving KC<sup>-/-</sup> mice exhibited a marked improvement in their condition over the course of the week. The significant reduction in the kinetics and magnitude of LPS-induced mortality in KC<sup>-/-</sup> mice clearly suggests a role for KC in this process.

**Biochemical analysis of indices of liver injury.** Among the pathophysiologic sequelae of systemic exposure to LPS is multiple-organ failure, particularly in the liver and lungs. To assess whether the enhanced survival of KC<sup>-/-</sup> mice was due to attenuation of liver injury, we measured two indices of hepatocellular damage, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), in the sera of KC<sup>+/+</sup> and KC<sup>-/-</sup> mice 12 hrs after administration of LPS (35 mg/kg) (Table VIII). While serum AST and ALT levels were elevated above baseline in both KC<sup>+/+</sup> and KC<sup>-/-</sup> mice after administration of LPS, there was a significant reduction in the fold-increase of AST levels in the sera of KC<sup>-/-</sup> mice (~2-fold) compared to KC<sup>+/+</sup> mice (~4-fold). The reduced elevation of serum AST in KC<sup>-/-</sup> mice suggests that KC contributes, at least partially, to liver injury following exposure to LPS.

**Evaluation of circulating leukocytes and neutrophil infiltration into tissue.** To determine whether the attenuation of liver injury was attributable to reduced inflammatory cell recruitment from the circulation into host tissue, we monitored circulating leukocyte counts and neutrophil recruitment into lung and liver in KC<sup>+/+</sup> and KC<sup>-/-</sup> mice after administration of LPS (35 mg/kg). Systemic exposure

Table VIII. Serum AST and ALT levels in KC<sup>+/+</sup> and KC<sup>-/-</sup> mice following injection with a lethal dose of LPS

Treatment <sup>a</sup>	AST (U/L) <sup>b</sup>		ALT (U/L)	
	KC <sup>+/+</sup>	KC <sup>-/-</sup>	KC <sup>+/+</sup>	KC <sup>-/-</sup>
saline control	104 ± 20	113 ± 32	86 ± 5	73 ± 3
LPS, 12 h	445 ± 42	251 ± 16 <sup>c</sup>	109 ± 9	117 ± 4

<sup>a</sup> Mice were injected i.p. with 35 mg/kg LPS.

<sup>b</sup> Data are represented as the mean ± SEM from 5 mice. This experiment is representative of two independent experiments.

<sup>c</sup> Data from KC<sup>-/-</sup> mice were significantly lower ( $p \leq 0.002$ ) than KC<sup>+/+</sup> mice.

to LPS is typified by a rapid and substantial decrease in peripheral leukocytes (69, 323). The reduction in total white blood cell (WBC) counts in both KC<sup>+/+</sup> and KC<sup>-/-</sup> mice at 6 and 12 h after LPS administration is clearly demonstrated in Table IX. However, the percent reduction in total WBC counts remained roughly equivalent at 6 and 12 h in KC<sup>+/+</sup> mice (41% and 44%, respectively), while in KC<sup>-/-</sup> mice, the percent reduction in total WBC counts (33% and 17%, respectively) was less sustained and almost restored to normal levels by 12 h. Accompanying the leukopenia after LPS exposure was a dramatic decrease in circulating lymphocytes that was paralleled by an increase in the number of neutrophils in both KC<sup>+/+</sup> and KC<sup>-/-</sup> mice. Of particular interest was that the increase in neutrophil counts in KC<sup>-/-</sup> mice ( $5.6 \times 10^3/\text{mm}^3$ ) was 2-fold higher at 12 h after LPS when compared to KC<sup>+/+</sup> mice ( $2.7 \times 10^3/\text{mm}^3$ ). No significant differences in the numbers of circulating lymphocytes, monocytes, eosinophils and basophils were observed (Table IX and data not shown).

We next examined myeloperoxidase (MPO) activity in lung and liver after administration of LPS as a quantitative measure of neutrophil accumulation within host tissue (324). In lungs of KC<sup>+/+</sup> and KC<sup>-/-</sup> mice, MPO activity increased dramatically within 1 h of LPS (~45-fold and 82-fold, respectively), decreased by almost 50% at 3 h, and then remained elevated above baseline for 12 h (Figure 20). Although the kinetic profiles were comparable, MPO activity was significantly higher in lungs of KC<sup>-/-</sup> mice at all time points when compared

Table IX. Effects of a lethal dose of LPS on circulating leukocyte populations in KC<sup>+/+</sup> and KC<sup>-/-</sup> mice

Treatment <sup>a</sup>	Cell Count ( $10^3/\text{mm}^3$ ) <sup>b</sup>							
	Total WBCs <sup>c</sup>		Neutrophils		Lymphocytes		Monocytes	
	KC <sup>+/+</sup>	KC <sup>-/-</sup>	KC <sup>+/+</sup>	KC <sup>-/-</sup>	KC <sup>+/+</sup>	KC <sup>-/-</sup>	KC <sup>+/+</sup>	KC <sup>-/-</sup>
saline	6.8 ± 0.5	8.1 ± 0.8	0.8 ± 0.1	1.0 ± 0.1	5.5 ± 0.5	6.2 ± 0.8	0.2 ± 0.02	0.2 ± 0.03
LPS, 6 h	4.0 ± 0.7	5.4 ± 0.8	2.4 ± 0.2	3.3 ± 0.4	1.5 ± 0.7	1.0 ± 0.4	0.1 ± 0.01	0.3 ± 0.13
LPS, 12 h	3.8 ± 0.9	6.7 ± 0.9 <sup>d</sup>	2.7 ± 0.7	5.6 ± 0.8 <sup>d</sup>	1.0 ± 0.7	0.4 ± 0.1	0.04 ± 0.01	0.3 ± 0.06

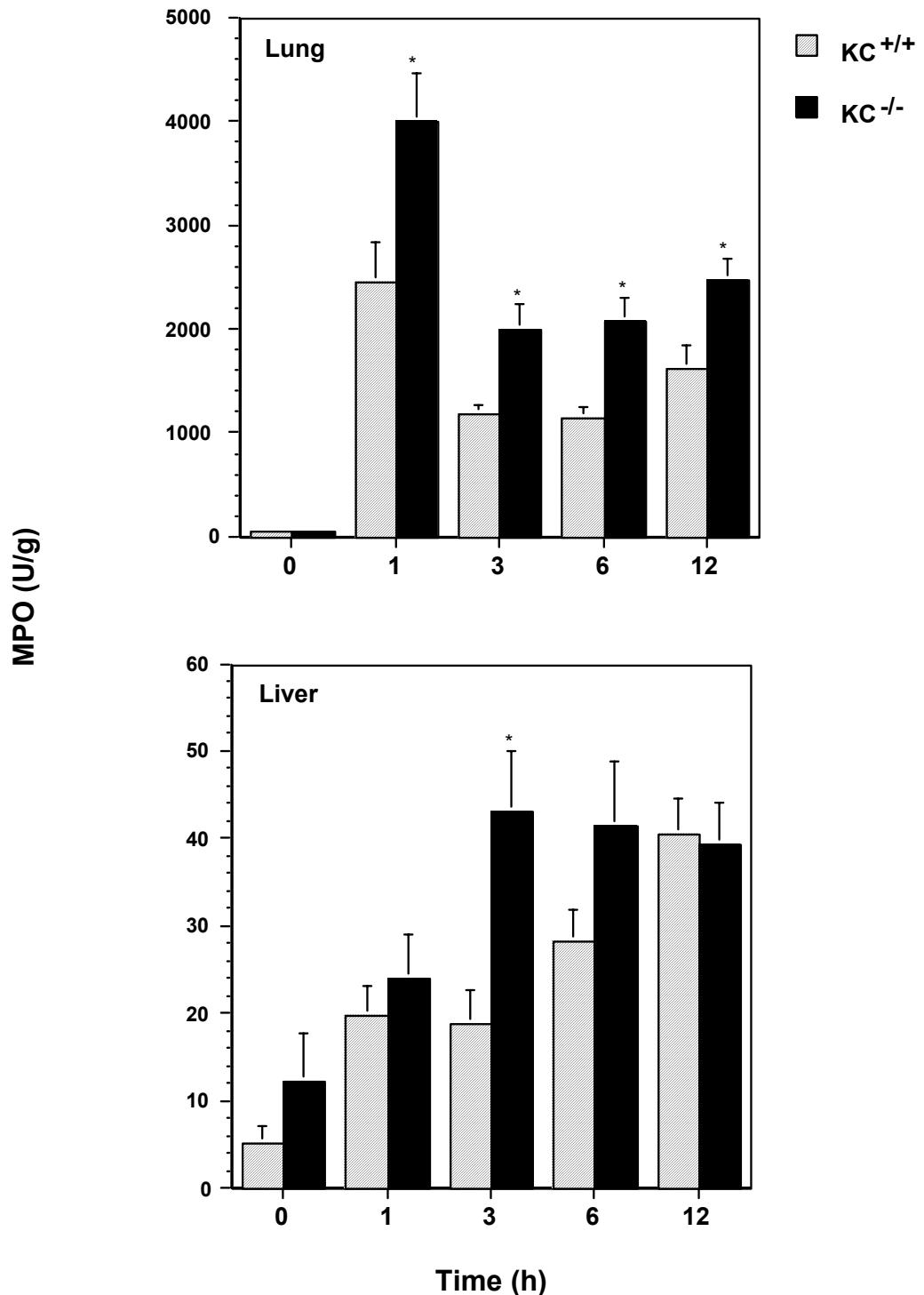
<sup>a</sup> Mice were injected i.p. with 35 mg/kg LPS.

<sup>b</sup> Data are represented as the mean ± SEM from 5-10 mice.

<sup>c</sup> Total WBC count also includes basophils and eosinophils which are not shown in this table.

<sup>d</sup> Data from KC<sup>-/-</sup> mice were significantly different ( $p \leq 0.05$ ) from KC<sup>+/+</sup> mice.

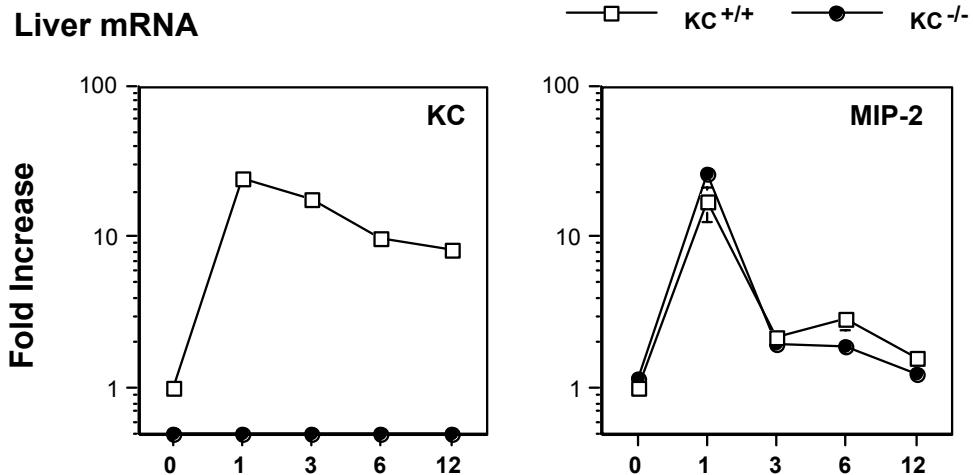
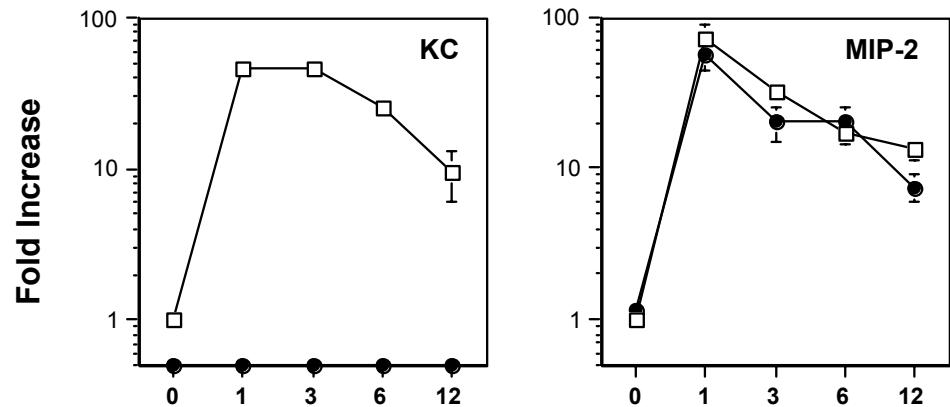
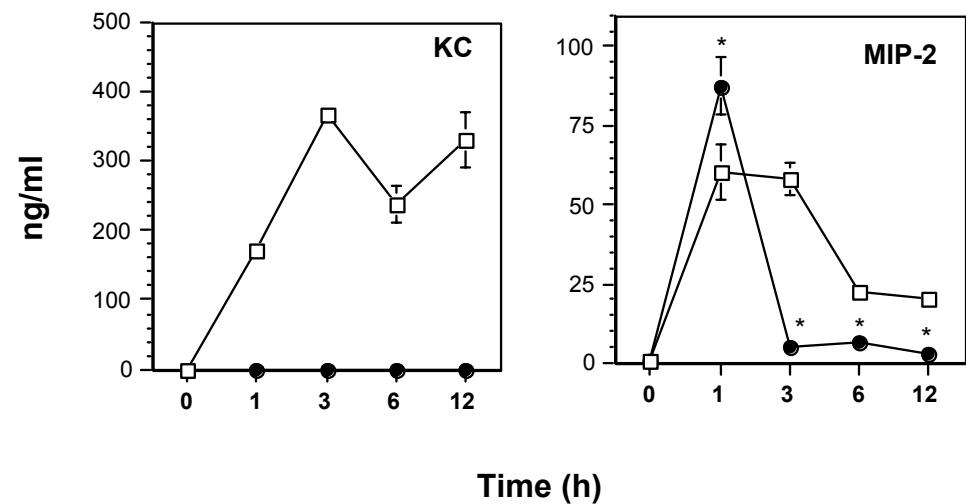
**Figure 20.** Neutrophil sequestration in lung and liver of KC<sup>+/+</sup> and KC<sup>-/-</sup> mice challenged i.p. with a lethal dose of LPS (35 mg/kg). Myeloperoxidase (MPO) activity is expressed in units per gram of tissue as described in *Materials and Methods*. Data are expressed as the mean  $\pm$  SEM from a total of 9 individual mice per time point from two independent experiments. An asterisk indicates MPO levels in KC<sup>-/-</sup> mice are significantly higher ( $p < 0.05$ ) than levels in KC<sup>+/+</sup> mice.



to KC<sup>+/+</sup> mice ( $p < 0.05$ ). In contrast to the lungs, MPO activity in the liver increased more gradually, with peak levels achieved 3 h after LPS exposure in KC<sup>-/-</sup> mice while levels in KC<sup>+/+</sup> mice remained substantially lower until 12 h. Taken together, these data suggest that the attenuation of liver injury was not attributable to reduced inflammatory cell recruitment into host tissue since the quantity of circulating and marginated neutrophils was actually enhanced, rather than diminished, in KC<sup>-/-</sup> mice. Furthermore, the neutrophilia observed in the circulation as well as in the lungs and livers of KC<sup>-/-</sup> mice may implicate a role for KC in the negative regulation of neutrophil production in normal mice.

**Kinetic analysis of the predominant neutrophil chemoattractants, KC and MIP-2, after administration of LPS.** To gain insights into the roles of KC after systemic exposure to LPS, we sought to establish the kinetics of KC mRNA and protein in KC<sup>+/+</sup> mice following LPS administration (35 mg/kg). As illustrated in Figure 21A and B, KC mRNA expression in livers and lungs of KC<sup>+/+</sup> mice was induced maximally over baseline as early as 1 h after LPS and remained sustained for 12 h. Likewise, KC protein was rapidly and substantially elevated in the serum within 1 h, peaked at 3 h, and remained elevated 12 h after LPS administration (Figure 21C). As would be expected from mice with a homozygous deletion of the KC gene, neither KC mRNA nor KC protein was detected in KC<sup>-/-</sup> mice. These data confirm and extend those shown in Figure 18C.

**Figure 21.** Kinetics of KC and MIP-2 mRNA expression in liver (A) and lung (B) and protein in serum (C) of  $KC^{+/+}$  and  $KC^{-/-}$  mice challenged i.p. with a lethal dose of LPS (35 mg/kg). Data are expressed as the mean  $\pm$  SEM from a total of 9 individual mice from two independent experiments. The means for mRNA fold induction are expressed relative to the response of untreated  $KC^{+/+}$  mice ( $t = 0$ ), which are assigned a value of 1. An asterisk indicates MIP-2 levels in the serum of  $KC^{-/-}$  mice are significantly different ( $p < 0.05$ ) than levels in  $KC^{+/+}$  mice. When not visible, error bars are smaller than the symbol.

**A. Liver mRNA****B. Lung mRNA****C. Serum Protein**

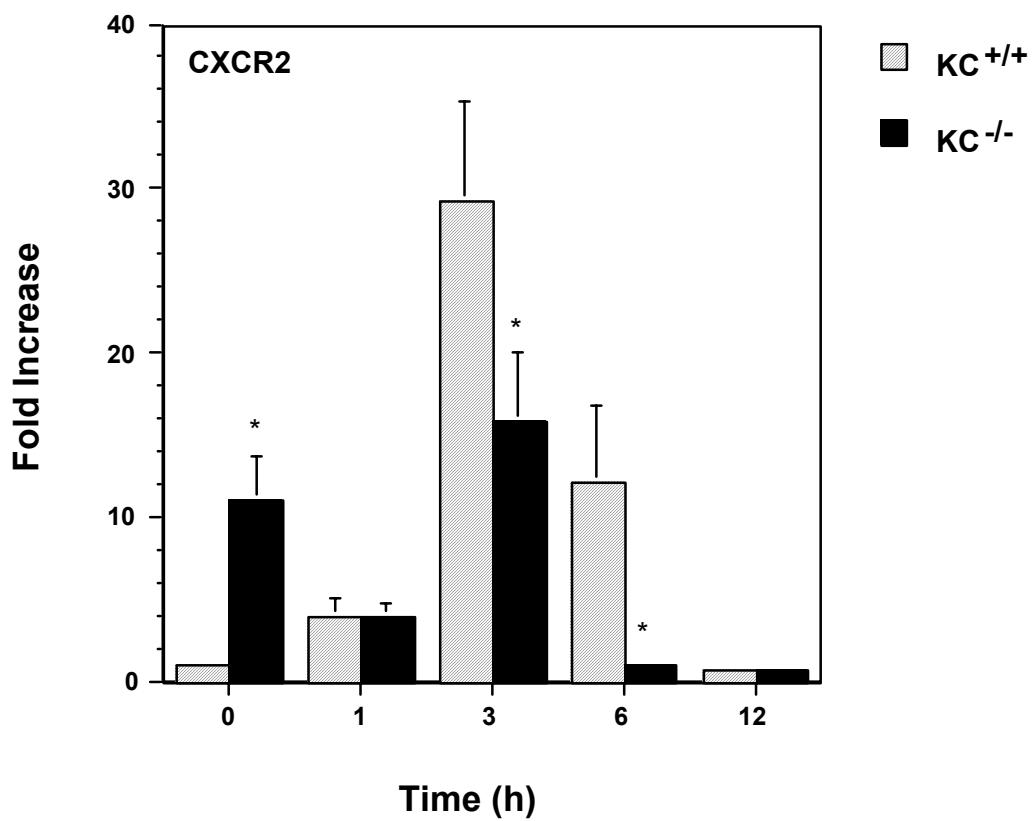
MIP-2 is another predominant neutrophil chemoattractant associated with acute inflammatory responses. Because of its functional redundancy with KC (118, 119), we evaluated the expression of MIP-2 mRNA and protein after administration of LPS to determine whether MIP-2 was altered in the absence of KC as a compensatory mechanism. Similar to KC, MIP-2 mRNA was rapidly and substantially induced over baseline within 1 h of administration of LPS in liver and lung of KC<sup>+/+</sup> and KC<sup>-/-</sup> mice, however, the levels were less sustained in the liver over 12 h (Figure 21A and B). In both liver and lung, the kinetic profiles of MIP-2 mRNA expression were remarkably comparable between KC<sup>+/+</sup> and KC<sup>-/-</sup> mice. In contrast to mRNA expression, the pattern of MIP-2 protein expression in the serum was significantly different in KC<sup>+/+</sup> and KC<sup>-/-</sup> mice (Figure 21C). In the serum of KC<sup>-/-</sup> mice, MIP-2 increased at 1 h (~88-fold) after LPS, then dropped precipitously to baseline levels by 3 h. In contrast, circulating MIP-2 levels increased substantially for 1 to 3 h (~60-fold) and then remained elevated over baseline levels for 12 h (~20-fold) in KC<sup>+/+</sup> mice. Collectively, these data indicate that the absence of KC does not alter MIP-2 expression at the level of mRNA in the liver and lung, yet it does alter the magnitude and kinetics of circulating MIP-2 in the serum in response to LPS.

KC and MIP-2, as well as other ELR<sup>+</sup> CXC chemokines, share the same chemokine receptor, the murine homologue of CXCR2 (122, 258). Unlike the human IL-8 receptors, CXCR1 and CXCR2, the murine homologue of CXCR2 is the only receptor known to bind these ligands in the mouse. While CXCR2 is

expressed predominantly on the surface of neutrophils, it has also been detected on monocytes, NK cells, and CD4<sup>+</sup> T cells (325, 326). Because of the functional significance of CXCR2 as the sole receptor for KC and MIP-2, we analyzed CXCR2 mRNA expression in the liver and lung of KC<sup>+/+</sup> and KC<sup>-/-</sup> mice after LPS. Due to the lack of availability of a commercial antibody to detect murine CXCR2, we did not evaluate CXCR2 expression on the surface of leukocytes. As shown in Figure 22, the basal level of CXCR2 mRNA expression is considerably higher in livers of untreated KC<sup>-/-</sup> mice when compared to KC<sup>+/+</sup> mice. However, hepatic CXCR2 mRNA levels were markedly reduced and less sustained in KC<sup>-/-</sup> mice at 3 and 6 h after LPS-challenge. In contrast, CXCR2 mRNA levels were not dysregulated in the lungs of LPS-challenged KC<sup>-/-</sup> mice (data not shown).

**Kinetic analysis of proinflammatory and anti-inflammatory cytokines after administration of LPS.** Among the key mediators of LPS-induced lethality are the proinflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$ , and IFN- $\gamma$ . To assess whether KC<sup>-/-</sup> mice were rendered more resistant to LPS-induced lethality due to a concomitant reduction in one or more of these major mediators, we analyzed their mRNA expression in livers and lungs, as well as circulating levels in KC<sup>+/+</sup> and KC<sup>-/-</sup> mice following administration of LPS. As expected, the mRNA levels of all three proinflammatory mediators were dramatically up-regulated within 1 h (TNF- $\alpha$  and IL-1 $\beta$ ) or 6 h (IFN- $\gamma$ ) after LPS in both liver and lungs of KC<sup>+/+</sup> and KC<sup>-/-</sup>

**Figure 22.** CXCR2 mRNA expression in the livers of LPS-challenged KC<sup>+/+</sup> and KC<sup>-/-</sup> mice. The mean fold induction of CXCR2 mRNA ± SEM (n = 9) after LPS-challenge (35 mg/kg) is expressed relative to the response of untreated KC<sup>+/+</sup> mice ( $t = 0$ ), which are assigned a value of 1. An asterisk indicates CXCR2 mRNA levels in the liver of KC<sup>-/-</sup> mice are significantly different ( $p < 0.05$ ) than levels in KC<sup>+/+</sup> mice. When not visible, error bars are smaller than the symbol.



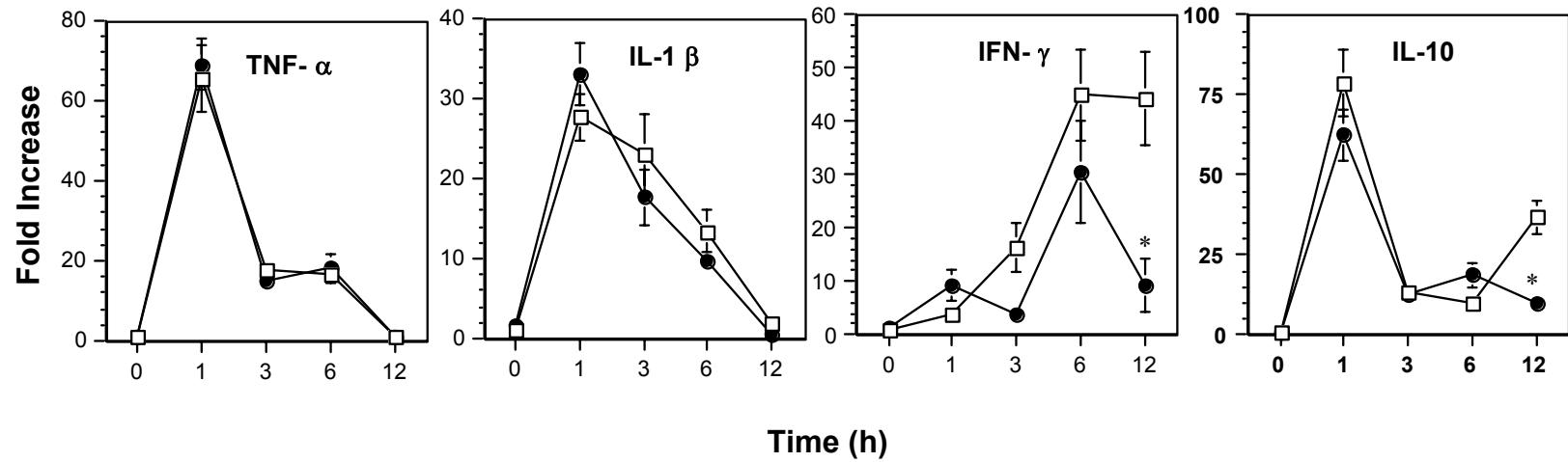
mice (Figure 23A and B). TNF- $\alpha$  and IL-1 $\beta$  mRNA levels were largely indistinguishable between KC $^{+/+}$  and KC $^{-/-}$  mice, except for slightly elevated levels of TNF- $\alpha$  in the lungs of KC $^{-/-}$  mice 1 h after LPS. In contrast, IFN- $\gamma$  mRNA levels were reduced in KC $^{-/-}$  mice at 12 h after LPS in liver and lungs. To further validate the results observed at the level of mRNA expression, we next examined TNF- $\alpha$  and IFN- $\gamma$  in the serum of KC $^{+/+}$  and KC $^{-/-}$  mice following administration of LPS. In accordance with the mRNA data, circulating levels of IFN- $\gamma$  were profoundly reduced in KC $^{-/-}$  mice at 6, 12, and 16 h after LPS (Figure 24). Additionally, circulating levels of TNF- $\alpha$  were substantially elevated in KC $^{-/-}$  mice at 1 h after LPS.

The anti-inflammatory cytokine, IL-10, has been shown to be protective in mouse models of endotoxemia and sepsis (285, 327-330). Further evidence is provided by the development of chronic intestinal inflammation in IL-10 knockout mice (331). To determine whether KC $^{-/-}$  mice were rendered more resistant to LPS-induced lethality due to a concomitant elevation in IL-10, we examined IL-10 mRNA expression in liver and lungs of KC $^{+/+}$  and KC $^{-/-}$  mice after LPS. In liver, IL-10 mRNA was rapidly and substantially induced in KC $^{+/+}$  and KC $^{-/-}$  mice (~78- and 62-fold, respectively) 1 h after LPS and then returned to baseline by 3 h (Figure 23A). However, by 12 h, the level of IL-10 mRNA in liver of KC $^{+/+}$  mice rebounded to ~37-fold increase over baseline, while remaining at baseline in KC $^{-/-}$  mice. Unlike the rapid elevation in liver, IL-10 mRNA levels in lungs increased more gradually after LPS, with the highest levels observed at 12

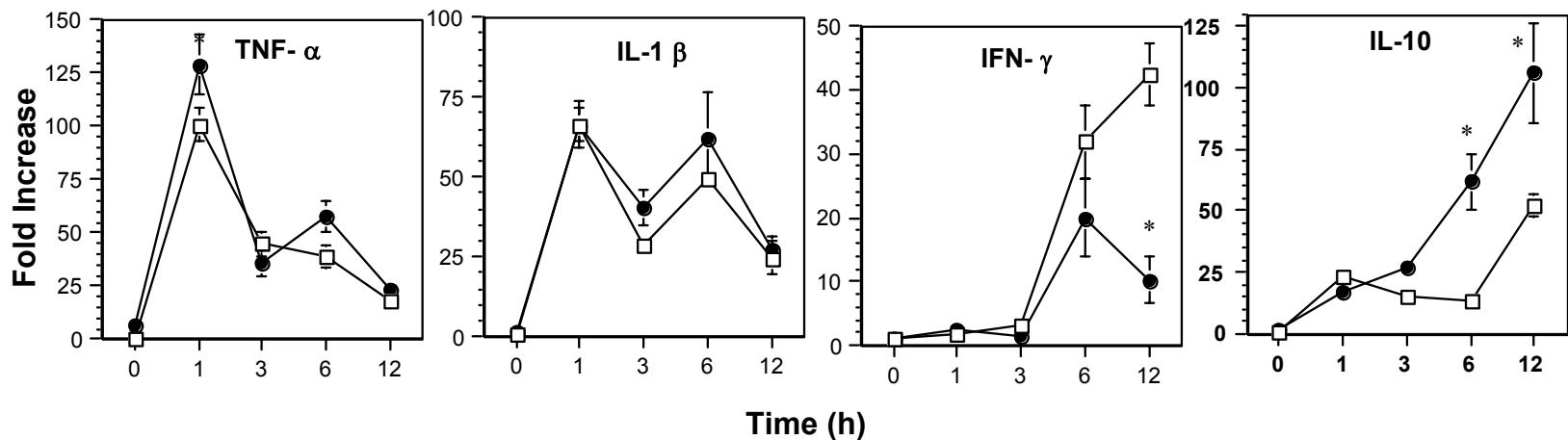
**Figure 23.** Kinetics of cytokine mRNA expression in the liver (A) and lung (B) of LPS-challenged (35 mg/kg) KC<sup>+/+</sup> and KC<sup>-/-</sup> mice. Data are expressed as the mean ± SEM from 9 individual mice per time point per strain from two independent experiments. The means are expressed relative to the response of untreated mice ( $t = 0$ ), which are assigned a value of 1. An asterisk indicates mRNA levels are significantly different ( $p < 0.05$ ) between KC<sup>-/-</sup> and KC<sup>+/+</sup> mice. When not visible, error bars are smaller than the symbol.

### A. Liver

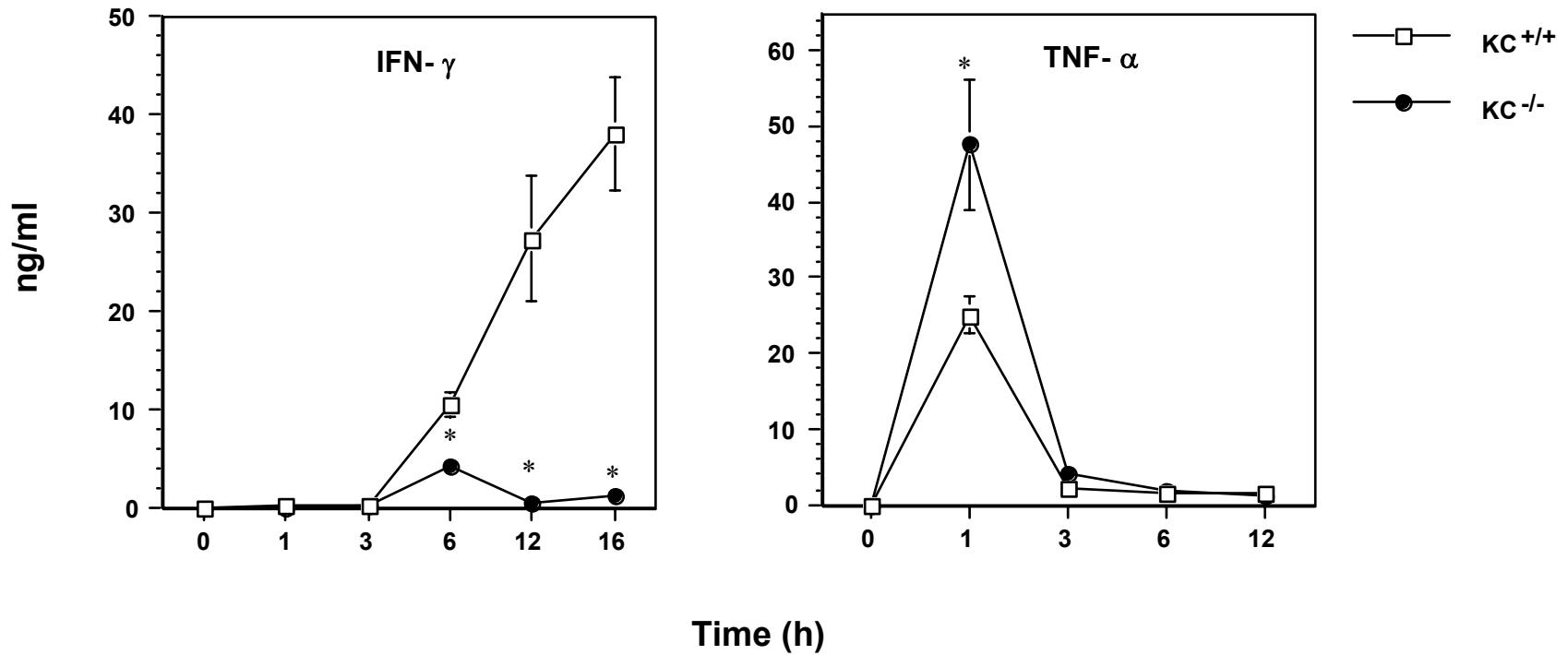
—□—  $KC^{+/+}$  —●—  $KC^{-/-}$



### B. Lung



**Figure 24.** Production of IFN- $\gamma$  and TNF- $\alpha$  in the serum of KC<sup>+/+</sup> and KC<sup>-/-</sup> mice after LPS-challenge. Mice were injected i.p. with either saline ( $t = 0$ ) or LPS (35 mg/kg) and serum was analyzed for cytokine protein production by ELISA. Data are expressed as the mean  $\pm$  SEM from 8-9 mice per time point from two independent experiments. An asterisk indicates protein levels are significantly different ( $p < 0.01$ ) between KC<sup>-/-</sup> and KC<sup>+/+</sup> mice. When not visible, error bars are smaller than the symbol.



h (Figure 23B). Of particular note was the significantly higher levels of IL-10 mRNA in lungs of KC<sup>-/-</sup> mice at 6 and 12 h (~62- and 106-fold, respectively) after LPS when compared to KC<sup>+/+</sup> mice (~13- and 52-fold, respectively). No commercial ELISA is available to measure IL-10 in the sera of these mice.

**Examination of upstream mediators of IFN- $\gamma$  production: IL-12, IL-18 and IL-15.** Like IL-12, IL-18 and IL-15 share the ability to induce IFN- $\gamma$  production in response to LPS (332-335). While these monokines are capable of stimulating IFN- $\gamma$  production individually, the synergistic cooperation of IL-18 or IL-15 with IL-12 potentiates IFN- $\gamma$  production (336, 337). Because of the reduction of IFN- $\gamma$  in KC<sup>-/-</sup> mice, we sought to determine whether upstream inducers of IFN- $\gamma$  were altered. To this end, we analyzed IL-15 mRNA expression in liver and lungs, as well as circulating levels of IL-12 and IL-18, in KC<sup>+/+</sup> and KC<sup>-/-</sup> mice following administration of LPS. We were unable to analyze circulating IL-15 levels in the serum due to the lack of availability of a commercial detection kit for IL-15. As shown in Figure 25, KC<sup>-/-</sup> mice produced 63-69% less hepatic IL-15 mRNA at 1 and 3 h, respectively, after LPS than KC<sup>+/+</sup> mice. Unlike the differences observed in the liver, pulmonary IL-15 mRNA was weakly induced to similar levels in both KC<sup>+/+</sup> and KC<sup>-/-</sup> mice at 3 and 6 h after LPS before plummeting below baseline levels at 12 h (Figure 25). In serum, bioactive IL-12 (p70) was 40-47% lower in KC<sup>+/+</sup> mice at 3 and 6 h, respectively, after LPS when compared to KC<sup>-/-</sup> mice (Table X). Likewise, both basal (0 h) and LPS-induced (1 and 3 h) levels of IL-18 were lower in KC<sup>+/+</sup> mice than KC<sup>-/-</sup> mice (Table X).

**Figure 25.** Kinetics of IL-15 mRNA expression in the liver and lungs of LPS-challenged (35 mg/kg) KC<sup>+/+</sup> and KC<sup>-/-</sup> mice. Data are expressed as the mean ± SEM from 9 individual mice per time point per strain from two independent experiments. The means are expressed relative to the response of untreated mice ( $t = 0$ ), which are assigned a value of 1. An asterisk indicates mRNA levels are significantly different ( $p < 0.05$ ) between KC<sup>-/-</sup> and KC<sup>+/+</sup> mice. When not visible, error bars are smaller than the symbol.

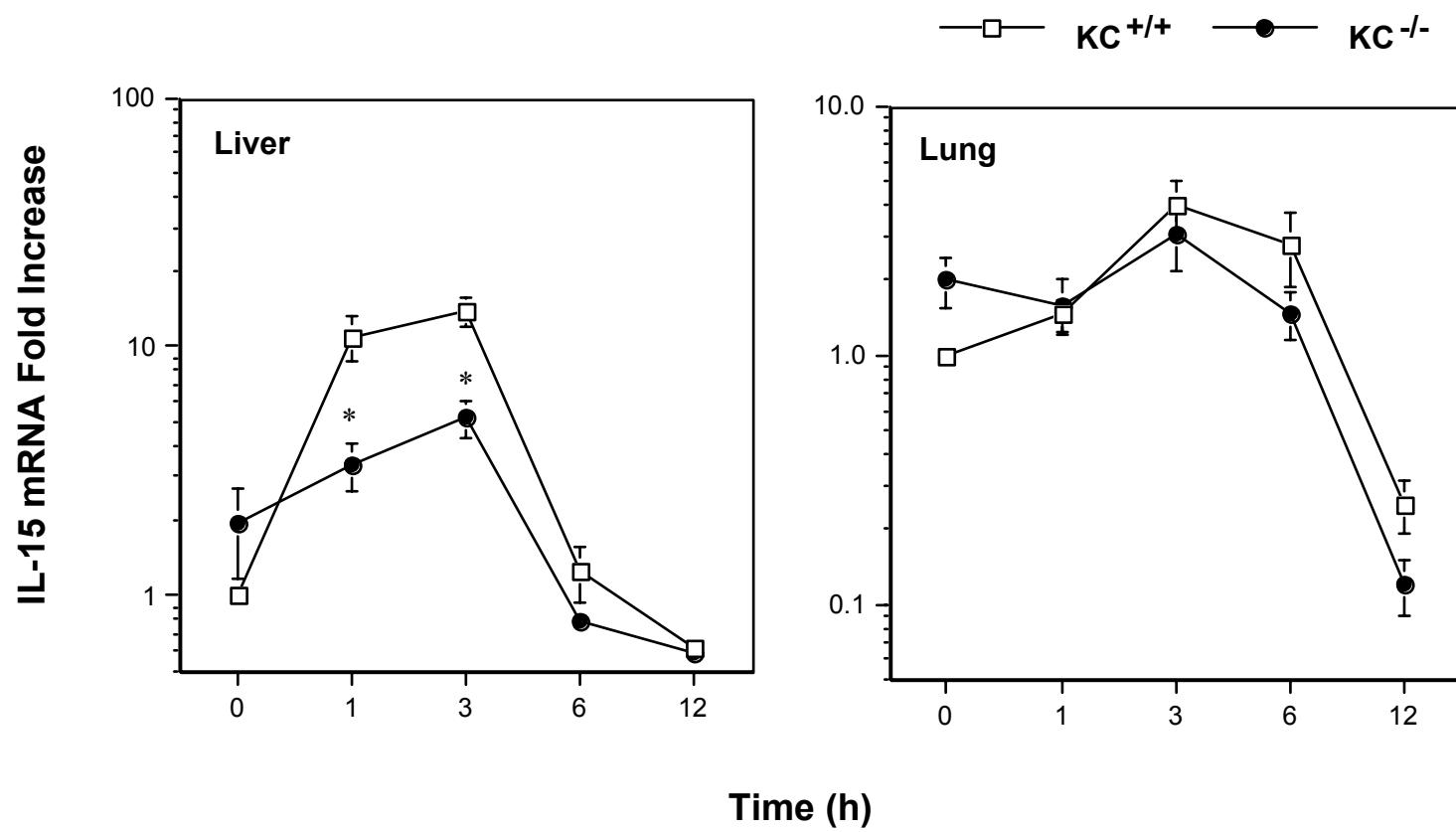


Table X. Circulating IL-12 p70 and IL-18 levels in KC<sup>+/+</sup> and KC<sup>-/-</sup> mice following injection with a lethal dose of LPS

Time <sup>a</sup> (h)	IL-12 p70 (pg/ml)		IL-18 (pg/ml)	
	KC <sup>+/+</sup>	KC <sup>-/-</sup>	KC <sup>+/+</sup>	KC <sup>-/-</sup>
0	ND	ND	86 ± 9	182 ± 30 <sup>c</sup>
1	ND	ND	145 ± 11	273 ± 27 <sup>c</sup>
3	957 ± 135	1,603 ± 243 <sup>c</sup>	164 ± 20	249 ± 9 <sup>c</sup>
6	1,543 ± 215	2,910 ± 394 <sup>d</sup>	ND	ND
12	235 ± 81	387 ± 90	ND	ND

<sup>a</sup> Time after i.p. injection of mice with 35 mg/kg LPS.

<sup>b</sup> Data are represented as the mean ± SEM from 4-9 mice.

<sup>c</sup> Data from KC<sup>-/-</sup> mice were significantly higher ( $p \leq 0.05$ ) than KC<sup>+/+</sup> mice.

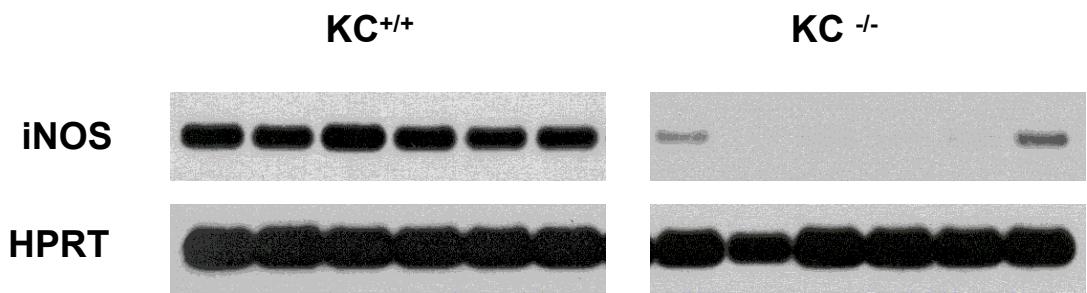
<sup>d</sup> Data from KC<sup>-/-</sup> mice were significantly higher ( $p \leq 0.005$ ) than KC<sup>+/+</sup> mice.

**Evaluation of inducible nitric oxide synthase, an IFN- $\gamma$ -dependent mediator of LPS-induced pathogenesis.** Excessive production of nitric oxide (NO) by the enzyme inducible nitric oxide synthase (iNOS) contributes to the circulatory failure observed in LPS-induced lethality (338, 339). Because NO production *in vivo* is dependent upon IFN- $\gamma$ , we sought to determine whether the reduction of IFN- $\gamma$  observed in KC<sup>-/-</sup> mice affected the levels of iNOS mRNA in the liver and lung and nitrate/nitrite levels in the circulation of KC<sup>-/-</sup> mice after LPS. As clearly depicted in Figure 26A, pulmonary iNOS mRNA levels were dramatically reduced in KC<sup>-/-</sup> mice at 6 and 12 h, respectively, after administration of LPS. Similar results were also observed for hepatic iNOS mRNA levels after LPS (data not shown). Consistent with iNOS mRNA, nitrate/nitrite levels in the sera of KC<sup>-/-</sup> mice were significantly reduced at 3, 6, and 12 h after LPS (Figure 26B). Together, these data suggest that the impaired IFN- $\gamma$  production in KC<sup>-/-</sup> mice results in the attenuation of an IFN- $\gamma$ -dependent mediator of endotoxicity, *e.g.*, iNOS.

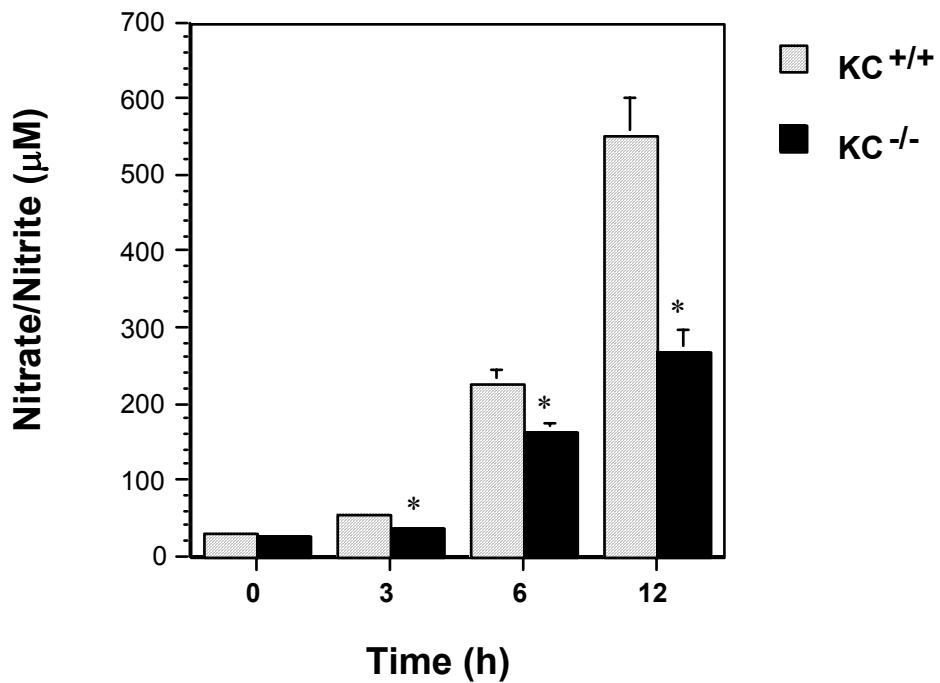
**Analysis of cell sources of IFN- $\gamma$ .** Classical NK and NKT cells have been reported to be the major producers of IFN- $\gamma$  in response to systemic exposure to LPS, while T cells play a very minor role (340, 341). To assess whether an alteration in IFN- $\gamma$ -producing cells accounted for the reduction of IFN- $\gamma$ , we measured the distribution of NK, NKT, and T cells in the livers of KC<sup>+/+</sup> and KC<sup>-/-</sup> mice following administration of LPS. Phenotypic analysis with respect to

**Figure 26.** Kinetics of iNOS mRNA expression in the lung (A) and serum nitrate/nitrite (B) of KC<sup>+/+</sup> and KC<sup>-/-</sup> mice challenged i.p. with a lethal dose of LPS (35 mg/kg). (A) Southern blots of iNOS mRNA expression are shown for 6 individual mice per group in lung at 12 h after LPS-challenge. (B) Serum nitrate/nitrite is expressed as the mean  $\pm$  SEM from 8-9 mice from two independent experiments. An asterisk indicates that serum nitrate/nitrite levels in KC<sup>-/-</sup> mice are significantly lower ( $p < 0.05$ ) than levels in KC<sup>+/+</sup> mice. When not visible, error bars are smaller than the symbol.

## A. Lung



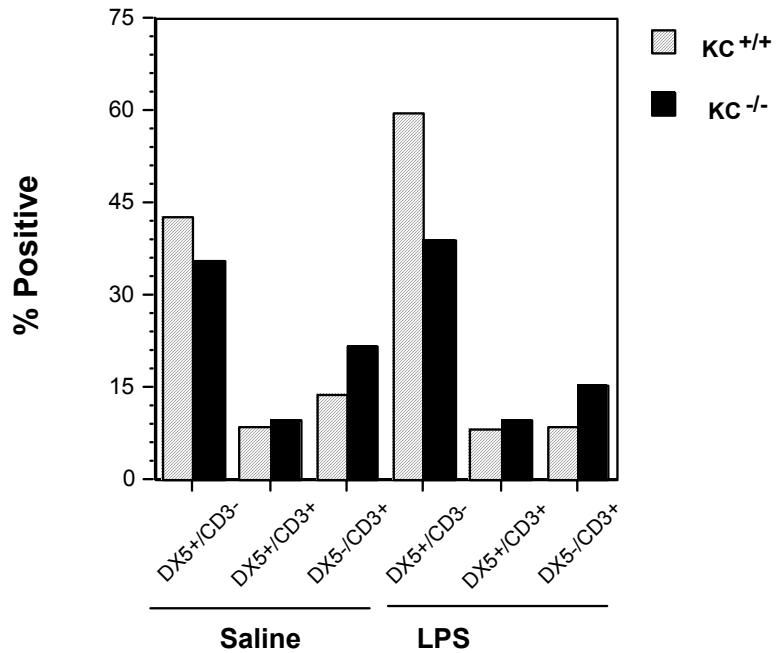
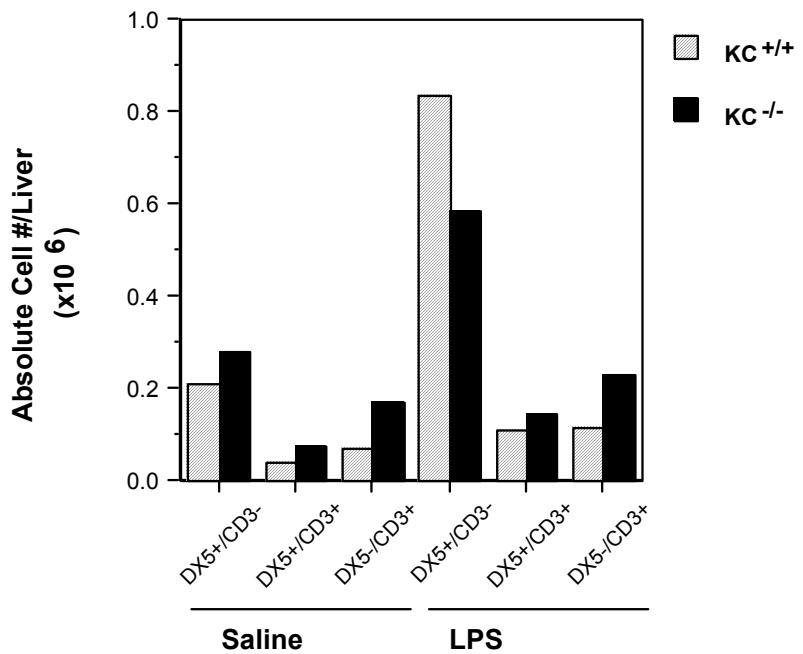
## B. Serum



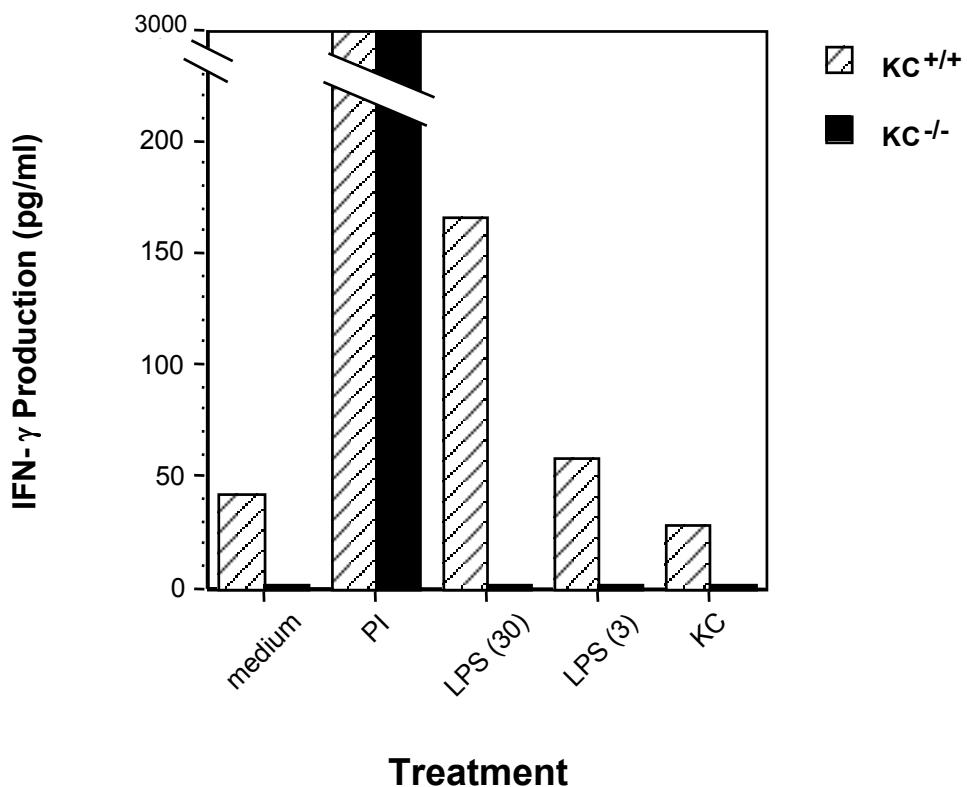
CD3 and DX5 expression was performed on hepatic leukocytes pooled from 3 mice per treatment group (saline or LPS). Figure 27 illustrates the percent distribution (A) and absolute cell number (B) of NK ( $\text{DX5}^+/\text{CD3}^-$ ), NKT ( $\text{DX5}^+/\text{CD3}^+$ ), and T ( $\text{DX5}^-/\text{CD3}^+$ ) cells of a representative experiment from two independent experiments. Overall, there were no differences in the percentages of NK, NKT, and T cell populations between  $\text{KC}^{+/+}$  and  $\text{KC}^{-/-}$  mice (Figure 27 and data not shown). NK cells ( $\text{DX5}^+/\text{CD3}^-$ ) accounted for the highest proportion of cells in livers of saline- and LPS-treated  $\text{KC}^{+/+}$  and  $\text{KC}^{-/-}$  mice, followed by T cells ( $\text{DX5}^-/\text{CD3}^+$ ), and then NKT cells ( $\text{DX5}^+/\text{CD3}^+$ ). The three cell populations were observed at roughly comparable levels in  $\text{KC}^{+/+}$  and  $\text{KC}^{-/-}$  mice, except for the decreased number of NK cells ( $\text{DX5}^+/\text{CD3}^-$ ) from LPS-treated livers (Figure 27B). However, this difference is considered negligible and is unlikely to account for the reduction of  $\text{IFN}-\gamma$  production in  $\text{KC}^{-/-}$  mice following administration of LPS (John R. Ortaldo, personal communication).

**IFN- $\gamma$  production by NK cells.** Although the quantity of cells capable of producing IFN- $\gamma$  was relatively unperturbed in  $\text{KC}^{-/-}$  mice, we sought to determine whether their ability to produce IFN- $\gamma$  in response to LPS remained. To this end, we enriched for NK cells from livers of untreated  $\text{KC}^{+/+}$  and  $\text{KC}^{-/-}$  mice by expansion in culture for 6 days with IL-2. Supernatants of NK cell-enriched cultures were harvested 24 h after culturing in the presence of phorbol myristate acetate and ionomycin (PI), LPS, or recombinant murine KC. As clearly depicted in Figure 28, the ability of NK cell-enriched cultures from  $\text{KC}^{-/-}$

**Figure 27.** Distribution of NK, NK/T, and T cells in the livers of  $KC^{+/+}$  and  $KC^{-/-}$  mice 6 h after LPS-challenge (35 mg/kg). Pooled leukocytes from livers of 3 mice per group were analyzed for expression of DX5 and CD3 by flow cytometry as described in *Materials and Methods*. The % positive (A) and absolute cell number per liver (B) of DX5<sup>+</sup>/CD3<sup>-</sup> (NK), DX5<sup>+</sup>/CD3<sup>+</sup> (NK/T), and DX5<sup>-</sup>/CD3<sup>+</sup> (T) are shown. This data is representative of two independent experiments.

**A.****B.**

**Figure 28.** Production of IFN- $\gamma$  in supernatants of NK cell-enriched cultures derived from livers of KC<sup>+/+</sup> and KC<sup>-/-</sup> mice. Pooled NK and T cells from livers of 3 mice per group were enriched for NK cells by expansion in culture with IL-2 as detailed in *Materials and Methods*. After culturing in complete RPMI-1640 medium alone or in the presence of 10 ng/ml of phorbol myristate acetate and 1 $\mu$ g/ml of ionomycin (PI), 30  $\mu$ g/ml LPS, 3  $\mu$ g/ml LPS, or 1  $\mu$ g/ml rKC for 24 h, cell-free supernatants were harvested and assayed for IFN- $\gamma$  by ELISA. The distribution of cell subsets was verified by flow cytometry as follows: KC<sup>+/+</sup> mice: 60% DX5<sup>+</sup>/CD3<sup>-</sup> (NK), 4% DX5<sup>+</sup>/CD3<sup>+</sup> (NK/T), 22% DX5<sup>-</sup>/CD3<sup>+</sup> (T); and KC<sup>-/-</sup> mice: 47% DX5<sup>+</sup>/CD3<sup>-</sup> (NK), 3% DX5<sup>+</sup>/CD3<sup>+</sup> (NK/T), 10% DX5<sup>-</sup>/CD3<sup>+</sup> (T). This data is representative of two independent experiments.



mice to produce IFN- $\gamma$  in response to LPS was completely abrogated. Furthermore, basal levels of IFN- $\gamma$  production was undetectable in cells derived from KC-/- mice. Only the nonspecific activators of intracellular signaling, phorbol myristate acetate and ionomycin (PI), was capable of circumventing the apparent signaling defect to induce IFN- $\gamma$  production. These data suggest that the IFN- $\gamma$  deficit in KC-/- mice is a result of the inability of IFN- $\gamma$ -producing cells to secrete IFN- $\gamma$  in response to specific stimuli, like LPS.

## DISCUSSION

### ***In Vitro Chemokine Regulation in Murine Peritoneal Macrophages***

A hallmark of the inflammatory response following systemic exposure to LPS is the highly coordinated recruitment of leukocytes into host tissues. This process is mediated, in part, by the secretion of chemokines at sites of incipient inflammation. Based upon *in vitro* studies, virtually every cell type has the potential to generate large quantities of several different chemokines. However, the pattern of chemokine expression *in vivo* appears to be much more selective. Furthermore, though many chemokines are expressed in inflamed tissues, individual chemokines have been demonstrated to play critical roles in endotoxemia and/or sepsis (71, 154, 173, 285, 342). However, to date, most studies have focused on the role of individual chemokines in this process. In the present study, we have taken a more comprehensive and systematic approach to identify cellular and molecular mechanisms that may provide for such selective chemokine expression and function in a model of endotoxicity.

Because the interaction of LPS with macrophages is pivotal in eliciting the proinflammatory cascade associated with sepsis (63, 286), we initially focused on characterizing the regulation of LPS-induced chemokine gene expression in macrophage cultures. However, the results of these studies cannot necessarily be extended to all populations of macrophages since these studies were conducted solely with thioglycollate-elicited peritoneal macrophages. While other studies

have noted similarities in the regulation of chemokine expression among different macrophage populations, significant disparity exists as well. VanOtteren *et al.* observed substantially higher levels of MIP-1 $\alpha$  production from alveolar macrophages when compared to peritoneal macrophages, yet treatment with dexamethasone or prostaglandin E<sub>2</sub> inhibited LPS-induced MIP-1 $\alpha$  expression equivalently in both macrophage populations (412). Likewise, LPS-induced MIP-2 protein production was reduced by dexamethasone treatment in both alveolar macrophages as well as in the murine macrophage cell line, RAW 264.7 cells (253). Additionally, while the effect of thioglycollate on peritoneal macrophages cannot be completely disregarded, we have consistently observed negligible levels of basal chemokine expression in uninduced cells.

One goal was to identify stimuli (both pro- and anti-inflammatory) *in vitro* that might account for differential expression of chemokines *in vivo*. Initially, we analyzed the kinetics and sensitivity of induction of CXC and CC chemokine mRNA expression in response to LPS and MPL, a nontoxic derivative of LPS. MPL was developed as an adjuvant for human vaccines (343) and a prophylactic drug for septic shock (344) due to its ability to function as both an immunostimulant and inducer of endotoxin tolerance without the toxic properties of LPS (287-291, 345). The reduced capacity of MPL to induce proinflammatory cytokines like TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-12, and IFN- $\gamma$  (289, 290, 292), in combination with its enhanced capacity to induce anti-inflammatory cytokines like IL-10 (292) may account, in part, for its attenuated toxicity. Since

tissue damage following exposure to LPS is related to the influx of recruited inflammatory cells, we assessed whether LPS and MPL differentially modulated chemokine induction *in vitro* from macrophages. Kinetic analysis indicates that in response to relatively high concentrations (200 ng/ml) of both LPS and MPL, chemokine mRNA expression was induced rapidly (0.5 h) for all genes except RANTES (2 h) and reached maximal levels within 1-2 (KC, MIP-1 $\beta$ , and MIP-2) to 6 h (IP-10, JE/MCP-1, MCP-5, MIP-1 $\alpha$ , and RANTES) (Figure 6). In contrast to the transient expression exhibited by most LPS-inducible early genes (274, 292, 346), chemokine steady-state mRNA levels remained elevated for 48 h. While these studies indicate that this panel of chemokine genes share the broad categorization as highly-inducible early genes, they do not share identical inductive patterns within a family nor within a functional group. KC and MIP-2 both exhibited early (1-2 h) peak levels of mRNA expression, consistent with their role in the recruitment of neutrophils, which are usually the first leukocyte population to arrive at a site of inflammation. Likewise, chemokines responsible for recruiting subsequent leukocyte infiltrates, *i.e.*, monocytes and T lymphocytes (*e.g.*, IP-10, JE/MCP-1, MCP-5, and RANTES), all exhibited delayed (6 h) peaks of mRNA expression. Thus, temporal regulation of macrophage chemokine expression may, in part, contribute to the sequence of inflammatory cell types that enter an inflamed site.

The kinetics of chemokine mRNA induction by MPL closely paralleled that induced by LPS. The high sensitivity of chemokine genes to induction by

LPS was demonstrated with concentrations as low as 0.1 ng/ml LPS and concentrations of 1-10 ng/ml LPS were sufficient for maximal mRNA expression (Figure 7). Whereas LPS and MPL exhibited similar kinetic profiles, dose-response analysis suggests that MPL was approximately 10-fold less potent than LPS for induction of chemokine mRNA expression, consistent with previous studies of MPL-induced gene expression (290, 292). However, at higher concentrations (10-1000 ng/ml), the inductive capacity of MPL was remarkably comparable to LPS.

Previous studies in our laboratory have established that dissociable signaling pathways exist in response to LPS (264, 294, 296). The approach we have used to dissect LPS-induced signaling pathways has taken advantage of the finding that the LPS-hyporesponsiveness of C3H/HeJ mice (*Lps<sup>d</sup>*) is exclusive for protein-free preparations of LPS as a result of a point mutation within the intracytoplasmic signaling domain of TLR4 (37, 38). In contrast to purified LPS, the butanol/water-extracted preparation of LPS, which contains bioactive endotoxin proteins (*i.e.*, but-LPS) (294), is capable of stimulating both *Lps<sup>n</sup>* and *Lps<sup>d</sup>* macrophages to induce a subset of LPS-inducible genes and tyrosine phosphorylation of MAP kinases (296). Recently, Hirschfeld *et al.* has demonstrated that the limited signaling capacity of protein-enriched preparations of LPS is attributable to signaling mediated through TLR2 in addition to TLR4 (297). Like endotoxin-associated proteins, a soluble extract of *T. gondii* tachyzoites (STAg) has been reported to elicit a similar response pattern

to that induced by endotoxin-associated proteins (263). In contrast to endotoxin proteins and STAg, the antitumor agent, MeXAA, induced the expression of a different subset of genes that was not associated with the tyrosine phosphorylation of MAP kinases (264). The results presented herein demonstrate that subsets of genes within both the CXC and CC subfamilies also exhibited different inducibility profiles in response to LPS, but-LPS, MeXAA, and STAg (Figure 8). Among the CXC chemokines, the two neutrophil chemoattractants, KC and MIP-2, shared identical profiles. Conversely, the CXC chemokine, IP-10, that has more “CC-like” biological functions, exhibited an inducibility profile shared by the CC chemokines, RANTES, MIP-1 $\alpha$ , MIP-1 $\beta$ , and JE/MCP-1. The profile of MCP-5 proved unique among the CC chemokines. MCP-5 was the only gene poorly induced by STAg in both *Lps<sup>n</sup>* and *Lps<sup>d</sup>* macrophages, and along with IP-10, was poorly induced in response to but-LPS in *Lps<sup>d</sup>* macrophages. In addition to emphasizing the differential modulation of this panel of chemokine genes, these results further substantiate our previous observations that dissociable signaling pathways exist that result in the expression of discrete subsets of genes in response to distinct stimuli.

Prior exposure to LPS induces a transient state of cellular hyporesponsiveness to subsequent stimulation with LPS known as endotoxin tolerance (300). It is believed that endotoxin tolerance is an adaptive mechanism to protect the host from further inflammatory injury in response to persisting bacteria and LPS. This generalized hyporesponsiveness has been postulated to

impair host immunity to secondary microbial infections. However, recent studies have demonstrated increased resistance to systemic infection with *Cryptococcus neoformans* or *Salmonella enterica* in endotoxin-tolerant mice, suggesting that host immunity remains intact (347, 348). While endotoxin-tolerant macrophages have a diminished capacity to produce inflammatory cytokines like TNF- $\alpha$ , IL-1 $\beta$ , IL-12, IP-10, and IFN- $\gamma$ , the production of other mediators like TNFRII, IL-10, and IL-1R antagonist is not suppressed (reviewed in 349). In this regard, cellular functions are not completely inhibited in endotoxin tolerance, but may be reprogrammed to adapt to chronic bacterial infection (302). In concordance with previous work in our laboratory that demonstrated a profound reduction of select chemokines in an *in vivo* model of endotoxin tolerance, we also observed a marked suppression of all chemokine mRNA expression and protein production in LPS-tolerized macrophages (except RANTES which could not be evaluated) (Figure 9 and Table II). In light of these observations, it is quite remarkable that antimicrobial immunity is maintained in the absence of chemokine production, since it is responsible for the directional migration of key phagocytic cells to sites of infection. Alternatively, if the reprogramming of cellular function in endotoxin tolerance is shifted towards an "anti-inflammatory" state, it is not unexpected that the regulation of chemokines is similar to that of other proinflammatory cytokines.

IL-10 is among the most potent anti-inflammatory agents induced in response to LPS (285, 327, 328, 330). The protective role of endogenous IL-10 in

endotoxicity and sepsis is underscored by the increased lethality following neutralization of IL-10 in mice (285, 330) and by the ability of exogenous IL-10 administration to prevent lethal shock in mice (327, 328). The anti-inflammatory action of IL-10 has been attributed to its ability to inhibit transcription, to promote degradation of mRNA, and/or to reduce translation of numerous proinflammatory cytokines and chemokines (303, 307, 308, 350, 351). However, it has been recently demonstrated that IL-10 may suppress gene expression in stimulus-selective fashion (352) and we wished to determine if differential sensitivity to IL-10 might be an important mechanism for generating differential expression of various chemokines. The LPS-induced chemokines previously reported to be sensitive to IL-10 inhibition include IP-10, KC, MIP-1 $\alpha$ , and MIP-1 $\beta$ . Our present findings confirm these observations and extend them by demonstrating that IL-10 also inhibits LPS-induced MIP-2, JE/MCP-1, MCP-5, and RANTES mRNA expression, although RANTES and MIP-1 $\beta$  were less sensitive to inhibition by IL-10, particularly at higher doses of LPS (Figure 10 and Table III). Collectively, these findings support a novel role for IL-10 as a key anti-inflammatory agent *in vivo*: as a result of the ability of IL-10 to inhibit the entire panel of 8 LPS-induced chemokine genes examined, one would predict that leukocyte infiltration (and subsequent release of proinflammatory mediators) into tissues would likely be attenuated.

Protein tyrosine phosphorylation pathways mediate many of the cellular effects of LPS and LPS-induced cytokines like TNF- $\alpha$  and IL-1 $\beta$  (28, 310-313).

Hence, specific protein tyrosine kinase inhibitors, like tyrphostins, have been evaluated for their ability to mitigate the effects of LPS. To date, tyrphostins AG126 and AG556 have been shown to reduce lethality in mouse, rat, and dog models of endotoxemia and/or sepsis (316, 353-356). The protection observed *in vivo* has been attributed to the ability of tyrphostins to inhibit LPS-induced production of TNF- $\alpha$  and NO (316). Our studies have demonstrated that AG556 is a potent inhibitor of LPS-induced JE/MCP-1 and MCP-5 mRNA and/or protein production, and to a lesser extent, IP-10 and RANTES (Figure 11 and Table IV). Interestingly, the inhibitory effects of AG556 were limited to those LPS-inducible chemokines that predominantly induce the directional migration of macrophages and T cells, but not neutrophils. Whether the suppression of these chemokines contributes to the reduced lethality observed *in vivo* requires further investigation. The mechanism of action of AG556 is not completely understood, but it does not inhibit the activation of the major LPS-induced tyrosine kinases, p38 and JNK (357), nor does it affect LPS-induced NF- $\kappa$ B nuclear translocation (358). However, the selective inhibition of this subset of chemokines suggests that AG556 likely mediates its inhibitory effects at a common target upstream of the LPS-induced transcriptional activation of these chemokines. This observation further demonstrates the selectivity of LPS-induced chemokine regulation and provides evidence for common pathways of induction that may contribute to coordinate regulation during the progression of an inflammatory response to LPS.

In contrast to the broad anti-inflammatory actions of IL-10, IFN- $\gamma$  is generally considered to be a potent macrophage activator that interacts synergistically with LPS to amplify the expression of inflammatory mediators like TNF- $\alpha$  and iNOS (273, 280, 317), in addition to enhancing LPS-induced lethality (359-361). LPS-induced MCP-5 and IP-10, genes which are inducible by IFN- $\gamma$  alone, and RANTES, were unaffected by co-treatment with LPS and IFN- $\gamma$  (Figure 12 and Table V). However, IFN- $\gamma$  has been shown to antagonize induction of JE/MCP-1, KC, MIP-1 $\alpha$ , and MIP-1 $\beta$  gene expression in LPS-stimulated macrophages (133, 309), and the present study extends this to include MIP-2. Thus, the differential action of IFN- $\gamma$  on individual chemokine genes provides an example of selective stimulus sensitivity that may be important in mediating the highly restricted patterns of chemokine gene expression noted *in vivo*.

The availability of mice in which transcription factors that mediate responses to IFNs have been deleted by gene targeting provides the opportunity to explore further the mechanisms involved in the regulation of LPS-induced chemokine expression. Activated STAT1 $\alpha$  homodimers (STAT1) are the primary transcriptional activation complex formed by IFN- $\gamma$ -stimulated cells and are necessary for the expression of IP-10 in response to IFN- $\gamma$  (362). Because IP-10 and MCP-5 are inducible with either IFN- $\gamma$  or LPS, we wished to determine if STAT1 was also necessary for chemokine expression in response to LPS.

Analysis of the LPS-inducibility of all of the chemokine genes examined in STAT1<sup>-/-</sup> macrophages revealed that only IP-10 and MCP-5 were strongly dependent on STAT1 for their induction by LPS (Figure 13). Recently, our laboratory identified the molecular basis for the STAT1-dependence of LPS-induced IP-10 and MCP-5 in a study by Toshchakov et al. (363). Their findings revealed that *E. coli* LPS, but not TLR2 agonists, strongly induces IFN- $\beta$  expression in a TLR4-dependent manner, which in turn, triggers STAT1 phosphorylation (363). Their observation that IP-10 and MCP-5 were not activated by TLR2 agonists suggested that IFN- $\beta$ -dependent STAT1 activation was necessary for their optimal expression (363). The ability of exogenous recombinant IFN- $\beta$  to restore the inducibility of IP-10 and MCP-5 in response to a TLR2 agonist further verified the IFN- $\beta$ -dependent STAT1 activation on their induction (363). Furthermore, TLR4-induced IFN- $\beta$  mRNA was demonstrated to be MyD88- and PKR (double-stranded RNA-dependent protein kinase)-independent, but TIRAP-dependent, and thus, IFN- $\beta$ -dependent IP-10 and MCP-5 mRNA expression is downstream of TIRAP (363).

Both IFN- $\gamma$  and LPS also activate expression of two other well-characterized IFN-responsive DNA binding proteins, IRF-1 and IRF-2 (274, 364). IRF-1 serves predominantly as a transcriptional activator (365), whereas IRF-2 functions generally as a transcriptional repressor (366-368). Although neither IRF-1 nor IRF-2 were required for induction of the chemokine genes examined, our studies also revealed a novel role for IRF-2 in the IFN- $\gamma$ -mediated inhibition

of LPS-induced KC, but not of other IFN- $\gamma$ -inhibitable genes (Figure 14 and Table VI). Promoter analysis of the murine KC gene has revealed two NF- $\kappa$ B motifs that allow for its induction by LPS; however, no functional IFN responsive regulatory sequences have been defined (369). Nevertheless, it is possible that the requirement for IRF-2 in negative transcriptional regulation of the KC gene by IFN- $\gamma$  is not mediated through interactions with an IFN-responsive sequence motif. In support of this possibility is the recent finding that both IRF-1 and IRF-2 can interact with NF- $\kappa$ B to control the transcriptional regulation of the MHC Class I gene (370), as well as others. Even though KC was the only LPS-inducible chemokine gene to exhibit IRF-2-dependent, IFN- $\gamma$ -mediated inhibition, it is not the only gene that we have found to exhibit this pattern of regulation: IFN- $\gamma$ -mediated suppression of LPS-induced IL-12 p35 mRNA expression is also derepressed in macrophages derived from IRF-2<sup>-/-</sup> mice (371). The molecular basis for the role of IRF-2 in this process will require further analysis. Nonetheless, the finding that no other IFN- $\gamma$ -inhibited, LPS-induced chemokine gene was regulated by either IRF-1 or IRF-2 further highlights the exquisite differential regulation of these genes.

Novel key findings of these *in vitro* studies include: 1) mRNA expression of all chemokine genes examined, except RANTES, was suppressed in LPS-tolerized macrophages; 2) IL-10 down-regulates LPS-induced mRNA expression for all chemokine genes examined; 3) the protein kinase inhibitor, AG556, selectively inhibits the mRNA expression of JE/MCP-1 and MCP-5; 4) IFN- $\gamma$

selectively inhibits LPS-induced KC, MIP-2, MIP-1 $\alpha$ , MIP-1 $\beta$ , MCP-1; 5) only induction of IP-10 and MCP-5 mRNA by LPS or LPS and IFN- $\gamma$  is Stat1-dependent; and 6) only IFN- $\gamma$ -mediated inhibition of KC expression was dependent upon IRF-2, but not IRF-1. Our findings demonstrate that individual chemokine genes are differentially regulated in response to potent inflammatory stimuli, such as LPS, despite their apparent redundant biological functions and intrafamily structural homology. Furthermore, when similarities in regulation were observed, the genes involved were not restricted to genes of like biological function or classification within a specific chemokine subfamily. These results suggest that individual chemokines fulfill unique roles during the sepsis cascade.

### ***In Vivo Chemokine Regulation in Response to LPS***

While our *in vitro* studies focused on the induction of chemokines as a result of the interaction of LPS with macrophages, the *in vivo* studies extended the analysis to encompass the broad array of cell types that participate in the physiologic response to LPS in the host. Multiple cell types (*e.g.*, leukocytes, endothelial cells, epithelial cells, fibroblasts, etc) (86) have been shown to produce chemokines in response to LPS; thus, measurements in serum and organs represent a composite of responding cell types that may have differential time courses of induction and differential LPS sensitivities. Our results demonstrate that the entire panel of CXC and CC chemokines, with the exception of RANTES, were also rapidly induced in the lung and liver after LPS

administration (Figure 15). The pattern of chemokine mRNA induction that we observed in the tissue following LPS exposure was accompanied by rapid and sustained production of chemokines (*i.e.*, JE/MCP-1, MIP-1, and MIP-2) in the serum (Figure 16). The observation that the kinetic profiles of chemokine production in the circulation did not completely parallel the corresponding mRNA expression in the tissue was not unexpected. Levels of circulating chemokines likely represent overflow from multiple host tissues as well as production from activated circulating leukocytes, and thus, will not necessarily reflect localized production of a chemokine in a particular tissue. While elevated serum chemokine levels may be required to sustain leukocyte mobilization from the circulation and the bone marrow over the duration of an inflammatory response, the profoundly elevated and sustained levels of JE/MCP-1 in the serum may reflect its role as an anti-inflammatory mediator (154). Although the influx of neutrophils precedes the recruitment of monocytes into tissues (71), our *in vivo* data do not support the hypothesis that this process is regulated by production of neutrophil chemoattractants before production of monocyte and lymphocyte chemoattractants as observed in our *in vitro* data. However, the physiologic relevance of the effects of chemokines is likewise dependent on the relative abundance of specific cell populations in the bloodstream, as well as on the corresponding up-regulation of other mediators of cellular trafficking (*e.g.*, adhesion molecules and chemokine receptors) to coordinate the time-dependent

and tissue-specific influx of inflammatory cells during the inflammatory response to LPS.

Previous studies in our laboratory revealed that hepatic macrophages (or Kupffer cells) were the primary cellular source of cytokines (*i.e.*, IL-1 $\beta$ , IL-6, IL-10, IL-12p40, and TNF- $\alpha$ ) in the liver of macrophage-depleted mice after administration of LPS (68). The selective depletion of hepatic macrophages by Cl<sub>2</sub>MBP-liposome treatment (68, 266, 272, 320) allowed us to assess the relative contribution of macrophages and/or macrophage-derived products in the induction of chemokine mRNA following exposure to LPS. To our knowledge, these studies provide the first direct evidence that liver macrophages contribute significantly to the induction of MIP-1 $\alpha$ , MIP-1 $\beta$ , RANTES, and MCP-5 mRNA following LPS (Figure 17). The observation that macrophages are the major source of certain chemokines is consistent with a previous report that identified macrophages as the primary source of MIP-1 $\alpha$  in the lungs of LPS-injected mice (71). Furthermore, MIP-1 $\alpha$ , MIP-1 $\beta$ , and RANTES are known to be induced by macrophage-derived products, such as TNF- $\alpha$  and/or IL-1 $\beta$ , in addition to LPS (105, 372). In fact, TNF- $\alpha$  has been reported to be a more potent inducer of RANTES than LPS (372). Thus, the induction of these chemokines by macrophage-derived products may account, in part, for their dependence on macrophages for their induction. Conversely, cell types apart from Kupffer cells produce IP-10, JE/MCP-1, KC, and MIP-2 mRNA by LPS in the liver of LPS-injected, macrophage-depleted mice. The observed elevated expression of IP-10,

JE/MCP-1, and MIP-2 mRNA in macrophage-depleted mice may reflect the absence of a negative regulator like IL-10, which is ablated in the liver by macrophage depletion (68).

In summary, we have identified differential patterns of chemokine expression that are temporally and spatially regulated *in vivo*. Additionally, hepatic expression of LPS-induced MIP-1 $\alpha$ , MIP-1 $\beta$ , MCP-5, and RANTES mRNA occurs predominantly in Kupffer cells, while IP-10, JE/MCP-1, KC, and MIP-2 expression occurs within other liver cell types. The specific pattern of expression of each individual chemokine is likely to reflect its unique function in the regulation and progression of an inflammatory response. Moreover, the contribution of chemokines to the inflammatory process that precedes the pathophysiologic sequelae of sepsis is evidenced by the profound elevation of serum chemokines as well as the upregulation of chemokine mRNA in the liver and lung, organs that typically succumb to dysfunction or failure in LPS-injected mice. Lastly, the complex orchestration of chemokines in response to LPS highlights the necessity for understanding the timing and localization of chemokine expression and production to identify potential targets for therapeutic intervention.

### **The Role of KC in a Murine Model of LPS-Induced Lethality**

In response to systemic exposure to LPS, the influx and activation of inflammatory leukocytes into host tissue, coupled with the overproduction of inflammatory mediators, is believed to underlie the tissue damage that precedes

multiple organ failure and death. To address the question of whether it would be feasible to target any individual chemokine as an approach to mitigating endotoxicity or sepsis, we took advantage of mice with a targeted disruption in the gene encoding the neutrophil chemoattractant, KC. Apart from the availability of these mice, KC was a reasonable chemokine to target because it is among the earliest and most highly expressed chemokines in organs susceptible to tissue injury in models of sepsis and endotoxemia (159, 253, 373, 374). This question is particularly relevant when considering the apparent functional redundancy exhibited *in vitro* among KC and the other LPS-inducible neutrophil chemoattractants, MIP-2, LIX, GCP-2, and MIP-1 $\alpha$  (71, 117, 118, 123, 253, 321, 322). Additionally, previous studies involving antibody neutralization of KC have been widely disparate in defining a role for KC *in vivo*. In a murine model of septic peritonitis, inhibition of KC had no effect on long-term survival or neutrophil sequestration in the liver, but it did reduce liver injury as measured by serum transaminases (375). Alternatively, antibody neutralization of KC markedly reduced the accumulation of neutrophils in the lungs and livers of rats after intratracheal or intravenous infusion of LPS, respectively (376-378). The varied outcomes of these studies are likely affected by the different experimental models of sepsis and endotoxemia, as well as the specificity, dosing, timing, and efficiency of the neutralizing antibody used. Therefore, the availability of KC knockout mice afforded us the unique opportunity to unequivocally define a role for KC in a model of LPS-induced lethality.

This study revealed that KC has a significant role in mediating LPS-induced lethality. To our knowledge, this is the first study to demonstrate a marked reduction in LPS-induced lethality in mice with a targeted disruption in a chemokine gene and suggests the potential applicability of this approach in sepsis. In addition to this study, chemokine receptor knockout mice, specifically CCR5 (receptor for MIP-1 $\alpha$ , MIP-1 $\beta$ , and RANTES)(248) and CCR4 (receptor for thymus and activation-regulated chemokine or TARC, and macrophage-derived chemokine or MDC)(379), have recently been shown to exhibit reduced mortality in response to LPS, suggesting a key role for CCR4 and CCR5 in mediating LPS-induced lethality. While not directly comparable, these studies provide evidence to support the hypothesis that components of the chemokine network are critical for mediating the inflammatory response to LPS. In our study, we found that administration of a lethal dose of LPS resulted in 100% mortality of KC $^{+/+}$  mice within 2 days, while the mortality of KC $^{-/-}$  mice progressed at a slower rate, with only 52% mortality after 7 days (Figure 19). Interestingly, KC $^{+/+}$  and KC $^{-/-}$  mice exhibited comparable symptomatology associated with early endotoxicity, like lethargy, piloerection, shivering (sign of fever), diarrhea, and watery eyes (a sign of enhanced vasopermeability), indicating that some of the causative inflammatory mediators were intact in KC $^{-/-}$  mice.

Because the influx of activated inflammatory leukocytes contributes to the tissue injury that precedes multiple organ failure and death, we initially predicted that the reduced mortality could be attributed to a reduction of

neutrophil infiltration into target tissue, and consequently, attenuation of organ failure. Tissue infiltration is preceded by a dramatic reduction in peripheral leukocytes, particularly neutrophils, in response to LPS exposure (69, 323). While a profound reduction in circulating neutrophils was observed in both KC<sup>+/+</sup> and KC<sup>-/-</sup> mice, the magnitude and duration of neutropenia in KC<sup>-/-</sup> mice was less severe and almost restored to normal levels by 12 h after LPS (Table IX). Repopulation of circulating neutrophils is accomplished through the recruitment of neutrophils from the bone marrow, the primary site of neutrophil generation and development, through an as yet undefined mechanism. Interestingly, the more rapid restoration of neutrophils into the circulation in KC<sup>-/-</sup> mice was consistent with the observation that the bone marrow of KC<sup>-/-</sup> mice contained elevated levels of neutrophils (Table VII). Similarly, mice lacking the receptor for CXCR2, the only known receptor for neutrophil chemoattractants in mice, exhibit profoundly elevated neutrophil levels in the bone marrow, as well as in the circulation, suggesting a potential role for CXC chemokines in the negative regulation of neutrophil production (258). The slight elevation of neutrophil levels in the bone marrow of KC<sup>-/-</sup> mice may well be sufficient for enhanced restorative capacity, but not compelling enough to implicate KC as the predominant CXC chemokine responsible for the negative regulation of neutrophil production. MIP-2 is the more likely candidate in the negative regulation of neutrophil production as it has been shown to exert CXCR2-mediated myelosuppressive activity (380). Instead, the more rapid restoration of

neutrophils in KC<sup>-/-</sup> mice may suggest a role for KC in limiting the recruitment of neutrophils from the bone marrow, possibly through CXCR2 receptor competition with the neutrophil chemoattractant responsible for initiating the recruitment.

Alternatively, neutrophils may be restored at a similar rate, but continued recruitment of neutrophils from the circulation into target organs may be disrupted in the absence of KC. Our findings do not support this possibility, and in fact, demonstrate a substantially greater accumulation of neutrophils into the lungs and liver of KC<sup>-/-</sup> mice in response to LPS (Figure 20). Instead, another neutrophil chemoattractant functions as the primary chemoattractant for the recruitment of neutrophils into target organ tissue or is capable of compensating for KC in its role as predominant neutrophil chemoattractant. We measured two indices of hepatocellular damage, AST and ALT, to assess whether the reduced mortality of KC<sup>-/-</sup> mice could be attributed to an attenuation of organ injury. The elevation of serum AST and ALT in both KC<sup>+/+</sup> and KC<sup>-/-</sup> mice following LPS-injection suggests the presence of cellular damage, primarily in the liver (413). While ALT is a more sensitive and specific indicator of acute liver injury, AST also serves as an indicator of damage to those tissues containing high concentrations of AST (*i.e.*, in decreasing order: liver, cardiac muscle, kidney, brain, pancreas, and lung)(413). Thus, the reduction of AST in the serum of KC<sup>-/-</sup> mice after LPS suggests a role for KC in the causation of cellular injury. However, further histological analysis would be necessary to identify whether

the liver is the primary source of AST in response to a lethal dose of LPS. Because there are no dramatic alterations in the gross pathology of the liver following administration of high dose LPS in the short duration of these studies (Dr. Cindy Salkowski, personal communication), it is difficult to substantiate "an attenuation" of liver injury as a result the reduced AST observed in KC<sup>-/-</sup> mice. Therefore, although the enhanced survival of KC<sup>-/-</sup> mice is consistent with a mitigated increase in AST, additional measures of liver injury will be required to substantiate this relationship. The significant reduction of AST, an indicator of hepatocellular damage, in the serum of KC<sup>-/-</sup> mice after LPS, suggests a role for KC in the causation of liver injury. The attenuation of liver injury, in turn, may have contributed to the enhanced survival of KC<sup>-/-</sup> mice after exposure to a lethal dose of LPS. Interestingly, the reduction in AST was achieved without an overall reduction in neutrophil accumulation in the liver. This finding is consistent with the study by Mercer-Jones et al., which demonstrated that neutralization of KC in a murine model of septic peritonitis had no effect on neutrophil sequestration in the liver, but did reduce liver injury as measured by serum transaminases (375). Previous studies have demonstrated that neutrophil-depletion protects liver and lung tissue from LPS-induced injury, implicating a critical role for neutrophils in mediating tissue injury (381, 382). However, neutrophil sequestration in the sinusoids alone is not sufficient to cause tissue damage (383). Instead, studies have shown that neutrophils must be appropriately activated to release proteases and reactive oxygen species to mediate liver injury (384, 385). For instance,

constitutive chemokine expression in KC and MCP-1 transgenic mice induced the infiltration of target leukocyte populations, but did not result in accompanying tissue injury (386, 149). Rather, administration of an additional stimulus (*i.e.*, LPS) was necessary for inflammatory activation of accumulated cells (149). In the absence of KC, neutrophils may not have received the appropriate activation signal to release mediators of tissue injury, thus accounting for reduced serum AST in KC<sup>-/-</sup> mice.

The observation that neutrophil accumulation into the lungs and liver was not disrupted in mice deficient in KC suggested the involvement of another neutrophil chemoattractant. Thus, we evaluated the temporal and organ-specific patterns of expression of MIP-2, another predominant neutrophil chemoattractant in acute inflammatory responses, to determine whether its expression coincided with neutrophil recruitment or was altered in the absence of KC as a compensatory mechanism. Like KC, MIP-2 is potently elicited by LPS to induce neutrophil chemotaxis, degranulation, and  $\beta_2$  integrin expression via binding to CXCR2 (118, 120, 122, 258). The contribution of MIP-2 in neutrophil recruitment during sepsis and endotoxemia has been demonstrated by antibody neutralization studies. In a murine model of septic peritonitis, neutralization of MIP-2 significantly reduced mortality and neutrophil recruitment into the peritoneal cavity (342). Similarly, Mercer-Jones et al. demonstrated that inhibition of MIP-2 attenuated sequestration of neutrophils in the lung, as well as in the peritoneum, during septic peritonitis (375). Additionally, neutrophil

accumulation in the lungs after airway instillation of LPS was substantially decreased following administration of anti-MIP-2 antibody (387). Our findings demonstrated that, like KC, MIP-2 mRNA expression in the lungs and liver, as well as circulating protein, was rapidly and profoundly induced after LPS exposure (Figure 21). However, MIP-2 mRNA expression in the liver and circulating MIP-2 were not sustained over time like KC, suggesting that MIP-2 may be more important in initiating acute inflammatory responses to LPS in those sites. The magnitude and sustained expression of MIP-2 mRNA levels in the lungs is consistent with previous studies that have demonstrated a more essential role for MIP-2 in neutrophil recruitment in the lungs during sepsis and endotoxemia (375, 387). The observation that the kinetic profile of MIP-2 coincides with the accumulation of neutrophils in the lungs and liver after LPS suggests that MIP-2 may be responsible for directing neutrophil migration into these target organs, but certainly does not confirm its involvement.

Administration of neutralizing antibodies to MIP-2 in LPS-injected KC<sup>-/-</sup> mice would be required to assess the contribution of MIP-2 in neutrophil accumulation in target organs.

Of particular note was the finding that circulating MIP-2 levels were markedly higher in KC<sup>-/-</sup> mice 1 hr after LPS, but dropped significantly lower than KC<sup>+/+</sup> mice from 3 to 12 hrs later. This observation underscores the physiological relevance of the functional redundancy inherent in the chemokine system, namely, that the dysregulation of one chemokine can affect the

expression of another functionally and structurally related chemokine. These findings are consistent with a previous study which demonstrated that lung-specific transgenic overexpression of KC in mice with Gram-negative bacterial pneumonia resulted in a concomitant reduction of MIP-2 levels in the lungs (388). The dysregulation of circulating MIP-2 levels, but not tissue mRNA levels, in KC<sup>-/-</sup> mice indicates a mechanism for coordinate regulation at the protein level, possibly through a regulatory feedback loop and/or receptor modulation. Our data provide evidence that KC contributes to tissue-specific receptor modulation. KC and MIP-2, as well as all other ELR<sup>+</sup> CXC chemokines, share the same chemokine receptor, CXCR2 (122, 258). While KC and MIP-2 have also been shown to bind the murine homologue of DARC, only binding to CXCR2 initiates signal transduction (389, 390). Our data demonstrated that basal levels of CXCR2 mRNA were significantly higher in liver, but not lungs, of untreated KC<sup>-/-</sup> mice (Figure 17). Interestingly, the observation that basal levels of CXCR2 mRNA were dysregulated only in the liver of KC<sup>-/-</sup> mice corresponds to the finding that KC is expressed constitutively in the liver, but not in the lungs, of wild-type mice. These data suggest that KC may negatively regulate basal CXCR2 expression only in tissues where its constitutive expression likely maintains basal trafficking of neutrophils. Besides affecting basal CXCR2 expression, CXCR2 mRNA levels were markedly reduced and less sustained in the livers of KC<sup>-/-</sup> mice, further demonstrating the impact of KC on CXCR2 modulation.

Evidence from numerous studies supports the paradigm that the outcome of endotoxemia or sepsis is associated with the balance of pro- and anti-inflammatory mediators. In a study by Walley et al., the increased mortality of septic mice, as a result of the inhibition of the anti-inflammatory mediator IL-10, was associated with elevated levels of the proinflammatory mediators TNF- $\alpha$  and IL-6 (330). Similarly, mice that were protected from LPS-induced lethality after administration of anti-MCP-1 antibodies exhibited reduced levels of the proinflammatory mediators TNF- $\alpha$  and IL-12 and enhanced levels of the anti-inflammatory mediator IL-10 (154). Likewise, the substantial reduction of the proinflammatory mediators TNF- $\alpha$ , IL-1 $\beta$  and IL-6 accompanied the enhanced resistance to LPS-induced lethality that was observed in CD14 knockout mice (32). Thus, the key proinflammatory mediators TNF- $\alpha$ , IL-1 $\beta$ , and IFN- $\gamma$ , and anti-inflammatory mediator IL-10, were evaluated to determine whether the reduction of LPS-induced lethality was associated with a concomitant alteration in one or more of these cytokines. TNF- $\alpha$  and IL-1 $\beta$  not only directly mediate, but also synergize, to produce a broad spectrum of pathophysiologic effects following exposure to LPS, to include the induction of hypoglycemia, prostaglandin-mediated fever, nitric oxide-mediated hypotension, leukocyte activation, and adhesion molecule up-regulation (61). While IL-1 $\beta$  levels in lungs and liver were relatively indistinguishable between KC $^{+/+}$  and KC $^{-/-}$  mice, circulating TNF- $\alpha$ , and to a lesser extent, TNF- $\alpha$  mRNA levels in the lungs of

KC<sup>-/-</sup> mice, were significantly higher than those in KC<sup>+/+</sup> mice 1 h after LPS exposure (Figures 23 and 24). The intact production of TNF- $\alpha$  and IL-1 $\beta$  in KC<sup>-/-</sup> mice likely accounts for the symptoms observed in these mice after LPS administration (*i.e.*, lethargy, piloerection, shivering, watery eyes, and diarrhea). Our observation that TNF- $\alpha$  levels were elevated in the absence of KC is consistent with previous studies that demonstrated that the administration of neutralizing antibodies to KC resulted in elevated TNF- $\alpha$  levels (376, 391). The contribution of KC in the negative regulation of TNF- $\alpha$  in these studies is not entirely clear, but may reflect an indirect effect of KC on the cells responsible for TNF- $\alpha$  production and/or TNF- $\alpha$  receptor modulation. In this study, the elevated levels of TNF- $\alpha$  in KC<sup>-/-</sup> mice may be attributable to the increased number of neutrophils in the tissues and circulation of KC<sup>-/-</sup> mice (Table IX and Figure 20). While not as significant a source of TNF- $\alpha$  as monocytes or macrophages, neutrophils have been shown to produce TNF- $\alpha$  in response to LPS (392). Further support for this notion is the temporal concordance between neutrophil influx into the tissues and TNF- $\alpha$  production, particularly in the lungs where the magnitude of infiltrating neutrophils is far greater than in the liver. The observation that elevated TNF- $\alpha$  levels did not result in enhanced lethality for KC<sup>-/-</sup> mice was not unexpected. Although significantly different, the levels of TNF- $\alpha$  were only 2-fold higher in KC<sup>-/-</sup> mice as compared to KC<sup>+/+</sup> mice. Additionally, the elevated levels of TNF- $\alpha$  were not sustained, but rather were only transiently expressed. Furthermore, the elevation in TNF- $\alpha$  levels were

accompanied by reduced levels of IFN- $\gamma$  (discussed below) and modestly enhanced IL-10 mRNA levels in the lungs in KC<sup>-/-</sup> mice as compared to KC<sup>+/+</sup> mice. Collectively, our data represents a balance of pro- and anti-inflammatory mediators that favors an attenuated outcome in response to a lethal dose of LPS.

As previously mentioned, the most profound difference between KC<sup>+/+</sup> and KC<sup>-/-</sup> mice was the dramatically reduced levels of circulating IFN- $\gamma$  and IFN- $\gamma$  mRNA in the lungs and liver of KC<sup>-/-</sup> mice after LPS exposure (Figures 23 and 24). This finding is particularly relevant since previous studies using neutralizing antibodies to IFN- $\gamma$  or mice deficient in IFN- $\gamma$  or IFN- $\gamma$  receptor have demonstrated improved survival after a lethal dose of LPS, implicating IFN- $\gamma$  as a pivotal mediator in LPS-induced lethality (359, 360, 393, 394). Additionally, IFN- $\gamma$  not only serves as a potent inducer of macrophage-derived proinflammatory mediators (*e.g.*, TNF- $\alpha$ , IL-1 $\beta$ , IL-12, nitric oxide, reactive oxygen intermediates), but also acts synergistically with several cytokines (*e.g.*, TNF- $\alpha$ , IL-1 $\beta$ ) to amplify the inflammatory response to LPS through diverse autocrine and paracrine networks (reviewed in 395). Furthermore, IFN- $\gamma$  enhances the sensitivity of macrophages and neutrophils to LPS through the up-regulation of TLR4 (396). Moreover, our data underscore the downstream consequences of impaired IFN- $\gamma$  production with the observation that inducible nitric oxide synthase, an IFN- $\gamma$ -dependent mediator of circulatory failure in endotoxemia, was greatly attenuated (Figure 26). Taken together, it is not unreasonable to suggest that the improved survival of KC<sup>-/-</sup> mice was largely

attributable to the substantial reduction in IFN- $\gamma$ . While the mechanism by which KC contributes to IFN- $\gamma$  production is unknown, there are numerous studies to support the involvement of chemokines and their receptors in the regulation of IFN- $\gamma$ . Studies involving mice with targeted deletions in the genes encoding the chemokines MIP-1 $\alpha$  and MCP-1, as well as the chemokine receptors CCR2 and CCR5, all reported profound reductions in IFN- $\gamma$  in response to Th1-dependent animal models (*i.e.*, *Mycobacterium bovis*, *Cryptococcus neoformans*, *Toxoplasma gondii*, *Leishmania donovani*, murine cytomegalovirus, experimental autoimmune encephalomyelitis) (397-403).

IL-12, IL-18, and IL-15 are known inducers of IFN- $\gamma$  production in response to LPS *in vivo* as evidenced by the substantial reduction in LPS-induced IFN- $\gamma$  levels in antibody neutralization or targeted gene deletion studies (332, 333, 335, 404). While all three cytokines have been shown to induce IFN- $\gamma$  production individually *in vitro*, the observation that antibody neutralization or targeted gene deletion of any one of these cytokines did not completely abrogate IFN- $\gamma$  production suggests that more than one inducer contributes to the overall IFN- $\gamma$  response. In fact, *in vitro* and *in vivo* studies have demonstrated that although IL-12 alone is the most potent inducer, it synergizes with IL-18 and IL-15 to potentiate IFN- $\gamma$  production greatly (335-337). Our data do not support the possibility that the reduction in IFN- $\gamma$  was attributable to defective production of the upstream inducers of IFN- $\gamma$ . Instead, we found that circulating levels of IL-12 and IL-18 were modestly elevated in KC $^{-/-}$  mice (Table X). While

the slight decrease in hepatic IL-15 mRNA may contribute to some reduction of IFN- $\gamma$  in KC $^{-/-}$  mice (Figure 25), the likelihood that this modest decrease accounts for the marked reduction in IFN- $\gamma$  production is remote when viewed in light of the concomitant increase in circulating IL-12 and IL-18. Although IL-12, IL-18, and IL-15 are capable of inducing IFN- $\gamma$  production in Th1, NK, and NKT cells, numerous studies have shown that NK and NKT cells are the predominant sources of IFN- $\gamma$  in response to LPS *in vivo* (340, 341, 405-407). In contrast, studies in athymic nude mice demonstrated that IFN- $\gamma$  production was largely unperturbed and lethality was unabated during lethal endotoxemia, suggesting that thymus-derived T cells were not significantly involved in mediating these responses (340). The contribution of NK and NKT cells has been mainly investigated in the generalized Shwartzman reaction, an experimental model of lethal endotoxemia resulting from two consecutive injections with low-dose LPS. Lethality in the Shwartzman reaction is dependent upon IFN- $\gamma$  production primarily by NKT cells in the priming or sensitization phase as evidenced by the reduced levels of IFN- $\gamma$ , and consequently, reduced lethality observed in NKT cell-depleted and NKT cell-deficient mice (340, 406). However, NKT cell-deficient mice were capable of IFN- $\gamma$  production and were not resistant to lethality following a single injection of high-dose LPS, suggesting that NK cells may serve as the predominant source of IFN- $\gamma$  in high-dose LPS models such as the one used in this study (406). Our analysis of NK, NKT, and T cell distribution in the liver where the largest population of NK and NKT cells

resides (408), indicates that these cell types were present in comparable proportions in KC<sup>-/-</sup> and KC<sup>+/+</sup> mice, except for the slightly decreased numbers of NK cells in livers from LPS-treated KC<sup>-/-</sup> mice (Figure 27). However, this difference in two independent experiments was negligible and unlikely to account for the dramatically impaired IFN- $\gamma$  production in KC<sup>-/-</sup> mice.

Since the number of IFN- $\gamma$ -producing cells was not grossly dysregulated in the absence of KC, we evaluated whether these cells were defective in their capacity to produce IFN- $\gamma$  in response to LPS in KC<sup>-/-</sup> mice. Our data indicate that NK cell-enriched populations from the livers of KC<sup>-/-</sup> mice were severely defective in LPS-induced production of IFN- $\gamma$  in a dose-dependent manner (Figure 28). Interestingly, the uninduced level of IFN- $\gamma$  was also abrogated in KC<sup>-/-</sup> mice. However, NK cell-enriched populations from KC<sup>-/-</sup> mice retained their ability to produce IFN- $\gamma$  as evidenced by the robust IFN- $\gamma$  response elicited by non-specific activators of protein kinase C (*i.e.*, phorbol myristate acetate) and calcium ionophores (*i.e.*, ionomycin). Clearly, these findings reveal a novel role for KC in the positive regulation of uninduced and LPS-induced IFN- $\gamma$  production. The mechanistic basis for KC in the regulation of IFN- $\gamma$  production is not apparent from the results of this study, and will require further investigation to delineate whether KC serves a direct or indirect role in the process. Because knockout studies involving other chemokines (*e.g.*, MIP-1 $\alpha$  and MCP-1) and chemokine receptors (*e.g.*, CCR2 and CCR5) also reported impaired IFN- $\gamma$  production (397-403), the underlying mechanism likely involves a common

component of chemokine receptor signaling rather than a component unique to KC-CXCR2 interactions. Additionally, our study did not include the evaluation of other upstream inducers of IFN- $\gamma$  production, like IL-12, IL-18, and IL-15, which may be more physiologically relevant in terms of the inducing stimuli *in vivo* and may offer more insights into the pathways involved in this defect. However, the observation that uninduced IFN- $\gamma$  production was ablated suggests that the defect extends beyond LPS induction alone. Although "uninduced" cells are not treated with an inducing agent for 24 hr prior to measurement of IFN- $\gamma$  in culture supernatants, it is important to consider that these cells have been enriched for NK cells by IL-2 stimulation for 6 days. Thus, the observation of ablated IFN- $\gamma$  production in "uninduced" cultures may be more reflective of the response of those cells to chronic stimulation with IL-2.

To pursue the mechanistic basis of the IFN- $\gamma$  defect in KC<sup>-/-</sup> mice further, it will be critical to eliminate the potential contribution of other genes that differ between the genetic backgrounds of the wild-type controls and the KC<sup>-/-</sup> mice to prevent misinterpretation of our findings. The derivation of the knockout mice used in this study involved the implantation of KC-deficient embryonic stem cells from 129/Sv mice into blastocysts of C57BL/6 pseudopregnant mice to produce chimeras. Germline transmission was determined by breeding chimeras with C57BL/6J, and then mice carrying the targeted deletion (heterozygous F1 progeny) were intercrossed to generate homozygous KC knockouts. C57BL/6J x 129/Sv F1 mice were used as control mice so that similar percentages of 129/Sv

background would allow for comparison to homozygous KC knockouts. While this strategy for knockout mice generation is an extremely common practice (409), it introduces the potential for unreliable data, and consequently, erroneous conclusions due to the contribution of genes that differ between 129/Sv and C57BL/6, apart from the one targeted for disruption. Therefore, to obviate the contribution of the 129/Sv background to the observed phenotype entirely, mice carrying the targeted mutation should be backcrossed a sufficient number of generations to C57BL/6J mice (*i.e.*, 7 times for 99.2% purity) (409). This issue is particularly relevant when considering the varying sensitivities of the respective inbred strains to LPS. Moreover, it is especially relevant when an NK cell phenotype is under scrutiny as the repertoire of critical Ly49 NK cell surface receptors is widely divergent when comparing the 129 to the C57BL/6 strain (410). Therefore, to provide a more rigorous test of the role of the genetic background, we are in the process of backcrossing mice carrying the KC deletion onto a pure C57BL/6 background (our most recent progeny have been backcrossed 8 generations). Upon completion, we intend to confirm my results presented herein and revisit the mechanism underlying the contribution of KC to defective IFN- $\gamma$  production in response to LPS in the backcrossed mice.

Collectively, our results demonstrate that KC is a significant contributor to the inflammatory response elicited by LPS. The apparent functional redundancies with other neutrophil chemoattractants cannot substitute for KC

function *in vivo*. However, its unique role is not contingent upon its ability to recruit neutrophils. Rather, KC appears to be necessary for the induction of IFN- $\gamma$  by NK cells, rather than its well-characterized chemotactic ability. It is tempting to speculate that KC may prime NK cells for increased IFN- $\gamma$ -production in response to LPS. If so, our results would represent the first example of chemokine priming of a cell type for the subsequent production of IFN- $\gamma$ , a cytokine that is central to LPS-induced lethality.

### **Summary of Work and Future Perspectives of Chemokines as Targets for Therapeutic Intervention in Gram Negative Sepsis**

The overall goal of this work was to identify cellular and molecular mechanisms that contribute to the differential regulation of chemokine expression in response to LPS in an effort to enhance our understanding of the unique roles of individual chemokines in a disease process as complex as sepsis. Our *in vitro* examination of LPS-inducible chemokine genes revealed variability in the magnitude and kinetics of expression in response to LPS, as well as differential patterns of expression in response to various modulators of inflammation, endotoxin tolerance, and mediators of transcriptional regulation. When similarities in regulation were observed, the chemokine genes involved were not restricted to genes of like biological function or classification within a specific chemokine subfamily. These findings provide insight into the complexity of the regulatory mechanisms that may account for their selective

expression and function *in vivo*. Next, we observed a profound and rapid induction of chemokines that were temporally and spatially regulated following LPS exposure *in vivo*. Not only do these findings underscore the contribution of chemokines to the inflammatory process that precedes the pathophysiology of sepsis, but also indicate unique functions for these important soluble mediators in the regulation and progression of the inflammatory response during sepsis. Finally, our evaluation of mice with a targeted disruption in the gene encoding the neutrophil chemoattractant, KC, revealed a significant, and more importantly, non-redundant role for KC in mediating LPS-induced lethality. This study demonstrated the feasibility of targeting an individual chemokine as an approach to mitigating endotoxicity or sepsis. Collectively, this body of work illustrates the complex nature of chemokine regulation and supports the hypothesis that individual chemokines fulfill unique roles during the sepsis cascade.

A thorough understanding of the specificity of individual chemokines during sepsis can be exploited for the rational design of a therapeutic intervention. Chemokines and their receptors have long been considered promising targets for therapeutic intervention because they are some of the most potent mediators of leukocyte trafficking and activation that contribute to the injurious inflammatory cascade during sepsis. Therapeutic intervention targeted at the transient blockade of chemokines or their receptors might dissipate an otherwise overwhelming inflammatory response, thus permitting other

intervention measures to be more effective. Chemokine receptors are especially desirable targets due to the success of small molecule inhibitors of G protein coupled receptors (GPCRs) in the treatment of several diseases (411). In fact, greater than 30% of currently marketed drugs are modulators of GPCR function (411). Several studies in recent years have provided proof of concept for the use of chemokine receptor antagonists in diverse models of disease and inflammation (246). However, the ever-growing number of chemokines and their receptors, coupled with the complexity of their ligand-receptor interrelationships, pose a significant hurdle in target identification. Moreover, the fundamental differences in the chemokine system in mouse and human (*i.e.*, no murine homologue of human IL-8; no human homologue of murine MCP-5; etc.) warrant extra caution when extrapolating results obtained in murine models to humans, and thus, present an additional challenge for target selection.

Chemokine redundancy, as defined by receptor binding *in vitro*, was once thought to be a major drawback for therapeutic intervention. However, chemokine redundancy is not necessarily reflected *in vivo* where specificity is achieved by careful orchestration of the kinetics and magnitude of ligand and receptor expression in a tissue- and disease-specific manner. The findings presented herein have demonstrated the specificity associated with chemokine regulation in response to LPS, further validating the potential for chemokines and their receptors as targets for therapeutic intervention in sepsis.

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